THE METABOLISM OF ACETATE IN SHEEP AFTER INJECTION OF PHLORHIZIN

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(Accepted for publication 23rd May, 1950.)

The lower fatty acids produced by fermentation of carbohydrate are of prime importance in the metabolism of ruminants (Marston, 1939; Phillipson, 1948). The major proportion of the energy requirements of the sheep, for example, is probably met by the large amounts of acetic, propionic and butyric acids which arise from microbial dissimilation of carbohydrates in the paunch (Marston, 1948). There is little exact knowledge however, of their relative usefulness to the animal.

Acetate is known to be an important source of carbon atoms for a number of biological syntheses and although its relationship to the intermediary metabolism of fat and carbohydrate has been investigated, the exact course of its breakdown is not yet known (Bloch, 1947). The possible conversion of acetate into glucose has been studied in phlorhizinized dogs in the classical experiments of Ringer and Lusk (1910), and the early findings have been examined by Deuel and Milhorat (1928). No significant change in the ratio of dextrose to nitrogen (D/N ratio) was observed to follow the injection of acetate and so it might reasonably be assumed that acetate is not converted to glucose. More recent experiments of Lorber, Lifson and Wood (1945), and Buchanan, Hastings and Nesbitt (1943), suggest that acetate can provide carbon atoms for the glucose molecule, but it probably cannot by itself form hexose.

Large amounts of acetic acid are absorbed by the sheep and so the efficiency with which this compound is used as a source of energy is of prime importance in the physiological economy of this animal.

MATERIAL AND METHODS.

Mature Merino ewes *aet.* 3–6 years (weight 30–40 kg.) were placed in metabolism cages designed to facilitate collection of faeces and urine (Marston, 1935) and food was withheld. The majority of the animals during this period were drenched daily with 5 gm. calcium carbonate suspended in 30 ml. water to alleviate scouring which frequently supervenes on fasting. Phlorhizin suspended in olive oil (1 gm. in 7 ml. of oil) was injected on the third and fourth day of fast and 0·5 gm. in 3·5 ml. oil on subsequent days. Following the injection of phlor-

hizin, the urine was collected at the end of each twelve hours, the bladder being drained by a catheter. Samples were preserved by the addition of thymol and toluene. The total output of sugar, nitrogen and ketone bodies during each period was estimated. Sugar was estimated by the method of Cole (1933), nitrogen by Kjeldahl digestion followed by micro distillation in the Pregl apparatus, and ketones by the gravimetric method of Van Slyke (Peters and Van Slyke, 1932).

The D/N ratio was determined for 5 or 7 periods (i.e. over the 3–4 days subsequent to the initial injection of phlorhizin), the acetate then administered and the D/N ratio obtained for the following 3 or 4 periods. The so-called "extra sugar" excreted in the urine and the percentage of "conversion" were calculated according to the method of Lusk (Ringer, 1912).

The acetate on some occasions was delivered through a fistula into the rumen or abomasum (Jarrett,1948a) but more often it was given either by intra-peritoneal or intravenous injection. For the intra-peritoneal injection 25–30 gm. of acetic acid were neutralized to pH 7·0 with NaOH, diluted to 180–200 ml. and injected over a period of 3–4 minutes. When administered into the alimentary tract 30 gm. acetic acid in one litre were dripped in over a period of 6 hours.

The intravenous injection was made into the jugular vein through a fine plastic tube. A No. 14 gauge needle was inserted into the vein, and the tube threaded through the needle for a distance of 6–7 inches. The needle was removed leaving the tube *in situ*. The necessary connections were made as described for continuous intravenous infusions (Jarrett, 1948b). In most cases 30 gm. acetic acid diluted to one litre and adjusted to pH 5·0–5·8 with caustic soda were injected over a period of 8–12 hours, but a few experiments were done without any pH adjustment. Two animals, however, were maintained on a continuous saline drip (1 litre/12 hours) throughout the experimental period. The acetic acid, administered to these animals, was partly neutralized so that the same amount of sodium was received over each twelve-hourly period. This allowed for any possible alteration in the D/N ratio exerted by the sodium ions on the fluid balance of the animal. A 10 p.c. glucose solution was also administered to a few animals by the intravenous or intra-peritoneal route, and the subsequent D/N ratio determined.

Owing to the difficulty of ensuring adequate supplies of commercial phlorhizin the material used in the majority of the experiments was prepared from fresh apple bark. Young shoots were collected during the months of November and December, the bark stripped immediately and immersed in 50 p.c. alcohol, about 1 kg. of bark to 2 litres of alcohol, and boiled for about an hour. The liquor was strained through gauze and evaporated to half the original volume. An equal volume of hot water was added and the liquor brought to boiling again and treated with about 50 gm. of Norit "adsorbent extra." It was found necessary to use a good grade of charcoal, otherwise the phlorhizin failed to crystallize. The cleared solution was filtered while hot and placed in the refrigerator for 48 hours. The product was re-crystallized from alcohol according to the method of Lusk (1928).

RESULTS.

The subcutaneous injection of phlorhizin into forty sheep in good nutritive condition, from which food was withheld, led to the excretion in the urine of sugar and nitrogen in the ratio 2·7–3·0. Two animals in poorer condition treated similarly gave a ratio of 1·8. A constant ratio was attained after two days. Following the administration of acetate, alterations in the D/N ratio indicated somewhat equivocal results: the route of administration was an important factor.

On three occasions when 30 gm. of acetic acid were introduced through a fistula into the abomasum of two sheep no increase in the D/N ratio resulted. Acetate administered *via* rumen fistulae to five sheep likewise brought about no alteration in the D/N ratio, but in three cases a slight rise in the excretion of ketone bodies occurred during the subsequent period.

Of the fifteen animals which received acetate by intravenous injection, a significant rise in the D/N ratio occurred in only three of them indicating in these a quantitative conversion to "extra" sugar of 40 p.c., 30 p.c. and 27 p.c. of the acetic acid respectively. These three cases were from a group of eight animals which received, over a period of 10 hours, 30 gm. of acetic acid in 1 litre of water (pH 5·8). Two animals of this group received the same amount of acetic acid neutralized to pH 7·0 in 250 ml. administered over 10–15 minutes and two received a continuous drip of physiological saline throughout the experiment except for the period when acetate was administered.

The output of ketone bodies following the acetate injection was considerably increased although this increase was not so marked when an apparent conversion to "extra" sugar had occurred. The protocol of animal No. 32 shown in Table 1 is typical of this phenomenon.

TABLE 1.

Protocol of sheep No. 32 receiving acetate by intravenous drip.

Days starvation	Urine collection/12 hours				Treatment	"Extra" sugar gm./24 hrs.	P.c. "conversion" to sugar
	Sugar gm.	N_2 gm.	Ketones gm.	D/N			
3	9·9	3·7	0·46	2·7	1·0 l. saline intrav./12 hrs.		
	11·4	4·6	0·87	2·5	1·0 l. saline intrav./12 hrs.		
4	11·3	4·0	1·5	2·8	1·0 l. saline intrav./12 hrs.		
	12·0	4·3	2·7	2·8	1·0 l. saline intrav./12 hrs.		
5	14·4	4·8	2·4	3·0	1·0 l. saline intrav./12 hrs.		
	11·9	4·1	7·3	2·9	30 gm. acetic + 16 gm. NaOH/1·0 l./12 hrs.	2	<1
6	12·2	3·8	4·0	3·2	1·0 l. saline intrav./12 hrs.		
	10·0	3·9	3·1	2·6	1·0 l. saline intrav./12 hrs.		
7	21·7	5·0	1·8	4·4	20 gm. glucose/0·7 l./12 hrs.	10	50
	10·8	3·1	0·95	3·5	1·0 l. saline intrav./12 hrs.		
8	13·6	4·8	1·7	2·8	1·0 l. saline intrav./12 hrs.		

Three animals developed haemoglobinuria after about 8 gm. of acetic/300 ml. had been received by intravenous injection over a period of three hours. In these cases the experiment was abandoned and after a further ten hours the urine was free from blood; no subsequent abnormal condition was noted thereafter.

Preliminary experiments showed that following intraperitoneal injection of acetate there was a marked rise in the D/N ratio over the first twelve-hourly period with a return to the pre-acetate level in thirty-six hours. Seven out of eight animals showed a "conversion" to sugar of 30–40 p.c. of the acetate. A typical result obtained with animal No. 83 is shown in Table 2. It was possible, however, that the treatment stimulated the sympathetic system and led to mobilization of sugar reserves. The "extra" sugar excreted in the urine under such circumstances may thus have resulted from the "alarm reaction" rather than from the acetate.

TABLE 2.

Protocol of sheep No. 83 receiving acetate by intraperitoneal injection.

Days starvation	Urine collection/12 hours: Sugar gm.	N_2 gm.	Ketones gm.	D/N	Treatment	"Extra" sugar gm./24 hrs.	P.c. "conversion" to sugar
3	12·0	3·8	0·09	3·1			
	10·2	3·6	0·19	2·9			
4	12·2	4·3	0·35	2·8			
	14·0	4·4	0·43	3·2			
5	11·5	4·6	0·58	2·5			
	21·3	4·0	2·4	5·3	25 gm. acetic/180 ml. (adjusted to pH 7·0)	10	40
6	12·9	4·3	0·78	3·0			
	9·9	4·3	0·68	2·3			
7	13·0	5·2	0·39	2·5			

This factor was controlled in a series of twelve animals by the preliminary injection into the peritoneal cavity of a hypertonic saline solution. On all but one occasion when this was done the D/N ratio increased during the following twelve-hourly period, indicating an excretion of "extra" sugar equivalent in most cases to 8–10 gm., before the normal ratio again prevailed. Six of these animals were given a further injection of hypertonic saline solution; three of these (Nos. 98, 95, 26) gave a response equivalent to the first injection, while the other three (Nos. 25, 78, 88) gave a smaller rise than previously. A subsequent injection of acetate to four of these animals gave a rise in the D/N ratio in only one case (No. 78).

TABLE 3.

Protocol of sheep No. 92 receiving saline and acetate by intraperitoneal injection.

Days starvation	Urine collection/12 hours: Sugar gm.	N_2 gm.	Ketones gm.	D/N	Treatment	"Extra" sugar gm./24 hrs.	P.c. "conversion" to sugar
3	12·2	3·2	0·12	3·8			
	9·0	2·6	0·34	3·5			
4	13·0	3·6	0·61	3·7			
	10·4	3·2	1·2	3·3			
5	22·6	4·1	1·9	5·5	25 gm. NaCl/180 ml.	9	
	14·6	3·9	2·1	3·8			
6	18·5	4·0	7·2	4·6	25 gm. acetic + 16 gm. NaOH/150 ml.	5·3	21
	14·4	3·8	3·4	3·8			
7	13·7	3·9	3·9	3·4			
	13·5	3·2	9·0	4·2	25 gm. acetic + 16 gm. NaOH/150 ml.	2·2	9
8	14·9	4·3	5·8	3·4			

Of the remaining six animals, four (Nos. 10, 11, 30, 92) received an injection of acetate twenty-four hours after the preliminary saline, and two (Nos. 35, 53) received acetate after a preliminary glucose injection. In two cases (Nos. 30, 92) a further treatment with acetate was given. Although in three animals (Nos. 10, 30, 92) there was a rise in the D/N ratio indicating an apparent conversion to sugar of 20–30 p.c. of the acetate, in view of the fact that a second

injection of saline at this period usually caused some rise, it was impossible to be certain that a real conversion had taken place. The protocol of a typical experiment with animal No. 92 is shown in Table 3 and a summary of the group is given in Table 4.

TABLE 4.

Summary of animals receiving saline and acetate by intraperitoneal injection.

Period	Animals								
	No. 25			No. 95			No. 98		
	Ketone gm.	D/N	"Extra" sugar	Ketone gm.	D/N	"Extra" sugar	Ketone gm.	D/N	"Extra" sugar
Average pre-saline	0·60	2·7		—	2·9		1·0	2·9	
Saline injection (1)	1·2	4·8	10 gm.	—	3·0	Nil	0·9	4·2	6 gm.
	0·13	3·3		—	2·9		0·8	3·1	
Saline injection (2)	1·7	3·5	4 gm.	—	3·1	Nil	1·0	3·9	5 gm.
	1·2	3·5		—	2·8		0·6	3·3	
Acetate injection	8·0	3·2	Nil	—	2·7	Nil	4·0	2·9	Nil
	1·7	2·8		—	2·5		1·1	2·1	
	No. 78			No. 88			No. 26		
Average pre-saline	0·7	3·3		1·2	3·5		0·4	2·6	
Saline injection (1)	0·9	5·0	4 gm.	2·0	5·1	10 gm.	0·8	3·9	7 gm.
	1·1	3·2		3·7	4·3		0·9	2·7	
Saline injection (2)	1·5	3·6	Nil	2·5	4·3	4 gm.	1·2	3·4	6 gm.
	1·6	2·8		—	3·9		1·2	3·2	
	1·7								
Acetate injection	4·6	5·2	4 gm.						
	1·8	3·0							
	No. 10			No. 11			No. 30		
Average pre-saline	4·3	2·8		0·5	2·7		0·8	2·8	
Saline injection (1)	1·8	4·4	8 gm.	0·5	3·6	3 gm.	0·8	5·3	12 gm.
	3·5	2·8			2·6		0·5	5·9	
Acetate injection (1)	6·5	5·2		1·4	4·0		0·7	6·3	
	2·2	2·6	7 gm.	0·8	2·5	3 gm.	0·5	2·4	10 gm.
	2·1	2·9		0·7	2·6		1·3	2·4	
Acetate injection (2)							5·1	3·7	
							2·5	2·5	2 gm.
							1·9	2·3	
	No. 92			No. 35			No. 53		
Average pre-saline	0·9	3·5		2·3	2·8		1·1	2·8	
Saline injection (1)	1·9	5·5	9 gm.	1·5	4·2	* 10 gm.	0·6	4·9	* 10 gm.
	2·1	3·8		2·1	3·3		1·0	3·2	
Acetate injection (1)	7·2	4·6		7·3	4·0		3·8	5·2	
	3·4	3·8	5 gm.	4·5	3·3	6 gm.	1·4	3·7	10 gm.
	3·9	3·4		5·0	3·1		1·2	2·1	
Acetate injection (2)	9·0	4·2							
	5·8	3·4	2 gm.						
	3·4	3·4							

* Nos. 35 and 53 received a glucose injection in lieu of the preliminary saline.

In a few animals which received a glucose solution either by intravenous or intraperitoneal injection the "extra" sugar indicated a recovery of only 50 p.c. of the amount administered irrespective of whether the sugar was given before or after the preliminary saline injection.

The excretion of ketone bodies showed a more consistent trend. On those occasions when an excretion of "extra" sugar followed the intraperitoneal injection of saline the ketone body output remained at the average fasting level. However, the administration of sugar always produced a definite fall in urinary ketones whereas the injection of acetate usually produced a very decided rise.

The intraperitoneal injection of the hypertonic salt solution, the acetate, and to a less extent the glucose solution produced a marked reaction in the animal, which became excited and went into a tetany particularly in the hind limbs. This passed off after a couple of minutes and the animal then appeared normal. Diuresis, accompanied by a marked rise in water intake, was often present in the subsequent two periods.

DISCUSSION.

Although somewhat equivocal results were obtained following the administration of acetate, little glucogenic effect could be detected in most of the experiments. When the acetate was introduced *via* the intraperitoneal route, reactions possibly due to stimulation of the sympatho-adrenal system made the results anomalous and difficult to interpret.

The methods adopted were based on the procedure used by other workers (Ringer and Lusk, 1910; Deuel and Milhorat, 1928) when working with dogs. However, in the present study, more control periods were determined in order to establish a constant D/N ratio; and solutions of hypertonic saline were used to assess the response of the animal to the treatment. The fact that glucose administration yielded a recovery of only 50 p.c. and, in fact, gave approximately the same excretion of "extra" sugar as saline or acetate given under similar conditions, suggests that in so far as the intraperitoneal route is concerned the reaction of the sheep is of more significance than the nature of the substance administered.

It is possible that when acetate was introduced into the rumen either a dilution or absorption factor may have masked any effect of the acetate; but this does not necessarily apply to the abomasum. Previous work had shown that when a solution of sodium acetate was administered into the abomasum, rapid absorption followed with a marked elevation of volatile acids in the systemic blood (Jarrett, 1947).

The ketogenic effect of acetate when injected intravenously is indicated by the marked rise in ketone body excretion. It appears that any conversion to sugar, or sparing effect on protein and reduction in nitrogen excretion, is insignificant in the phlorhizinized sheep.

An analysis of Table 4 indicates that little, if any, excretion of "extra" sugar could be definitely attributed to the acetate *per se*. The initial injection,

irrespective of whether it was saline, glucose or acetate, on most occasions led to an excretion of "extra" sugar equivalent to approximately 8 gm. If acetate had been effective a greater increase than that attributable to the saline would be expected. Moreover, when acetate was given after the preliminary saline, the amount of sugar excreted was comparable with that excreted when a second saline injection was given. If an initial saline injection gave a response, then a similar response resulted from the further injection of either saline or acetate. On each occasion when acetate was given as a third injection, no "extra" sugar was excreted, but as the animals at this stage had been fasted for 8–9 days the conditions may have been unphysiological. The results therefore indicate that acetate is probably not converted to, and excreted as, sugar in the urine of phlorhizinized sheep.

The excretion of unchanged acetate in the urine is unlikely (Wisling, 1885; and von Knieriem, 1885), but the haemoglobinuria that developed in three of the animals indicated that some abnormal kidney effect may have occurred. The amount of acetate received before the haemoglobinuria developed was small compared with that absorbed from the rumen during active fermentation, but the pathological condition may have been influenced by the effect of the phlorhizin on the liver or kidney. Haemoglobinuria accompanied by the presence of albumin and cells in the urine has been reported by Sklodowski (1925) as a feature associated with acetate poisoning.

This work is in agreement with some preliminary experiments of Barcroft (1945) but does not confirm unpublished results of Hitchcock and Phillipson, from which Phillipson (1947) has reported that "with sheep the increase in excretion of glucose compared with that of nitrogen suggested that 30 to 40 p.c. of acetic acid may be converted to sugar."

The ketone-body excretion showed no significant rise following the saline or sugar injections, but a marked increase in the output of ketone bodies occurred after acetate. Although this proves that acetate administered to phlorhizinized sheep is ketogenic it does not necessarily prove direct conversion. The experiments of Gurin, Delluva and Wilson (1947) for example, showed that when isotopic lactate was administered to phlorhizinized rats approximately half of the radioactive carbon of the lactate was converted to fat and urinary ketone bodies although the anticipated amounts of "extra" urinary glucose were formed.

Considering the difficulties inherent in these experiments with phlorhizinized sheep many of the objections to the D/N ratio raised by Soskin (1941) would appear to be valid. Only very cautious conclusions can be drawn but it would appear that in the sheep, some of the "extra" sugar excreted may well arise from factors other than a direct conversion of the substances under investigation.

SUMMARY.

The variation of the amount of sugar, nitrogen and ketone bodies excreted in the urine was determined after the administration of acetate to phlorhizinized sheep.

The D/N ratio showed that little, if any, of the sugar excreted in the urine following acetate administration, could be attributed to a direct conversion of the acetate.

Although it proved unlikely that acetate was glucogenic a marked ketogenic effect was observed.

Acknowledgments. The authors gratefully acknowledge the help of Mr. G. B. Jones and colleagues for the nitrogen determinations, and are particularly indebted to Mr. H. R. Marston, F.R.S., Chief of the Division, for his constructive advice throughout.

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INTERFERENCE BETWEEN INACTIVE AND ACTIVE INFLUENZA VIRUSES IN THE CHICK EMBRYO

III. INHIBITOR OF VIRUS HAEMAGGLUTINATION IN THE CHORIO-ALLANTOIC MEMBRANE

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(Accepted for publication 6th June, 1950.)

A number of inhibitors of haemagglutination by influenza viruses has been described, e.g. the normal serum inhibitor (Francis, 1947), cyst mucoid (Burnet, McCrea and Anderson, 1947) and the inhibitor in normal allantoic fluid (Svedmyr, 1947). Many of these have been shown to be enzymically destroyed by influenza viruses and by the soluble enzyme, RDE, of *V. cholerae*. Friedewald *et al.* (1947) extracted inhibitor from various tissues, and Bovarnick and de Burgh (1947) prepared an extract from red blood cells which behaved in solution as a virus inhibitor. Hirst (1943) described cell receptors in the ferret lung, which adsorbed influenza virus, and similar receptors have been described in other tissues susceptible to influenza virus infection (Stone, 1947; Fazekas de St.Groth, 1948). These receptors, like the inhibitors, were found to be acted upon by viruses and RDE.

The present paper describes the characteristics of inhibitor extracted from the chorio-allantoic membranes of chick embryos, and the reduction in inhibitory titre occurring during the course of influenza virus infection in the allantoic cavity.

MATERIALS AND METHODS.

Virus Strains.

The following strains were used in the form of infected allantoic fluid, harvested 2 days after allantoic inoculation of 11-day chick embryos.

Influenza A: WSE, PR8, MEL, BEL.
Influenza B: LEE.

RDE.

An extract of the 4Z strain of *V. cholerae* containing the receptor-destroying enzyme (RDE) was prepared by the method of Burnet and Stone (1947). The preparation of RDE used in these experiments had a titre of 4,000 units/ml.

Normal saline. 9·0 gm. sodium chloride in 1,000 ml. distilled water.

Citrate-saline. 20 gm. sodium citrate in 1,000 ml. distilled water.

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Calcium-acetate-saline. Isotonic sodium acetate—acetic acid buffer adjusted to pH 6·2 and containing 0·1 p.c. calcium chloride.

Borate Buffer. Equal parts of M/20 sodium metaborate and M/5 boric acid (pH 8·5).

Fowl Red Cells.

Blood was obtained from the wing vein of adult white Leghorn fowls. Cells were washed in normal saline and made up to a 1 p.c. suspension in normal saline.

Haemagglutination Titration.

Serial twofold dilutions of virus (0·25 ml.) were prepared in normal saline and an equal volume of 1 p.c. fowl red cells was added to each dilution. The cells were allowed to settle at room temperature and readings were made by observing the pattern of sedimented cells. The end-point was taken as the amount of virus causing partial agglutination, this amount being one agglutinating dose (A.D.). The haemagglutinin titres are expressed as the reciprocal of the initial dilution of virus at the end-point.

Inhibitor Titration.

Serial dilutions of inhibitor were prepared in normal saline. An equal volume of virus containing 5 A.D. was added and the mixtures kept for 30 minutes at room temperature. A third volume of fowl cells was added and these were allowed to settle at room temperature. Partial agglutination indicated the end-point. Inhibitory titres are expressed as the reciprocal of the initial dilution of inhibitor present at the end-point.

Infectivity Titration.

Serial ten-fold dilutions of virus were prepared in 10 p.c. horse-serum saline. 0·05 ml. volumes were inoculated allantoically into 11- or 12-day chick embryos, 6 per group. After incubation for a further 42 hours at 35° C. the allantoic fluids were harvested and one drop added to 0·25 ml. of 1 p.c. fowl cells, growth of virus being detected by haemagglutination. The 50 p.c. infectivity end-point (ID_{50}) is the dilution of virus which will infect 50 p.c. of the inoculated eggs.

Preparation of Indicator Viruses.

The methods of Stone (1949a) were used to convert viruses into the inactive indicator state, in which their haemagglutinins are particularly sensitive to inhibition by mucoid inhibitors, and the viruses lack enzymic activity.

Heated LEE (H-LEE) — LEE virus heated as undiluted allantoic fluid at 56° C. for 30 minutes.

Treated PR8 (T-PR8), *Treated MEL (T-MEL)*, *Treated BEL (T-BEL)* — 6 parts of virus heated at 56° C. for 30 minutes in 2 parts of 2 p.c. citrate saline and 1 part of borate buffer.

Heated WSE (H-WSE) — Virus heated as undiluted allantoic fluid at 52° C. for 30 minutes.

Preparation of Interfering Viruses.

Viruses were inactivated for use as interfering agents by the methods described for indicator viruses except that they were heated for 60 minutes (to ensure loss of infectivity). Interfering viruses were tested for infectivity by inoculating 0·05 ml. (less than a minimal interfering dose) into 6 eggs. Only viruses which did not infect any of the eggs after 42 hours' incubation were used as interfering agents.

Preparation of Chorio-allantoic Membrane Suspension.

11- or 12-day old chick embryos were used throughout. After removal of the shell over the air-sac, the shell membrane and underlying chorio-allantoic tissue were cut and the contents of the egg discarded, leaving the chorio-allantoic membrane lining the shell. By this method the portion of the membrane covering the viscera was not included. The membrane was removed carefully and cleared of adhering mucinous material. A number of washings in normal saline removed most of the blood. The membrane was ground with alundum and suspended in 1 ml. of normal saline. After thorough mixing, the ground membrane was lightly centrifuged and the supernatant fluid used as the standard membrane suspension.

Interference Experiments.

Groups of six 11- or 12-day embryos were inoculated allantoically each with an interfering dose of inactive virus. If 0·5 ml. or greater volumes were injected an equal amount of allantoic fluid was first withdrawn. The eggs were incubated for one hour at 37° C. and then inoculated again with 0·05 ml. of varying dilutions of challenge virus. Controls were inoculated with challenge virus only. The eggs were then incubated for 42 hours at 35° C. when the allantoic fluids were harvested and titrated individually for their haemagglutinin content.

EXPERIMENTAL.

Virus Inhibitor in Suspensions of Ground Chorio-allantoic Membranes.

Chorio-allantoic membranes of 11- or 12-day chick embryos were tested for the presence of inhibitor of influenza viruses. A suspension of membrane was prepared (see Methods) and titrated for inhibitor of haemagglutination by LEE and MEL viruses, both heated and unheated. Table 1 shows typical inhibitory titres.

TABLE 1.

Inhibitor in suspension of ground chorio-allantoic membrane.

State of virus	Inhibitory titre	
	LEE	MEL
Active	18	30
Heated	480	750

Inhibitor titrated against 5 A.D. of each virus.

It can be seen that membrane suspension inhibits agglutination particularly by heated ("indicator") viruses. This behaviour is characteristic of inhibitors prevously described.

Normal membrane suspension did not inhibit infectivity of active virus. Dilutions 10^{-6}, 10^{-7}, and 10^{-8} of MEL virus were mixed with equal volumes of membrane suspension and with normal saline as a control, and allowed to stand for one hour. These mixtures were inoculated into groups of eggs and the 50 p.c. infectivity end-points were determined. Virus mixed with membrane suspension, and control virus both showed infectivity end-points of $10^{-7.8}$.

Heat Stability of Membrane Inhibitor.

When undiluted membrane suspension was heated at 65° C., a heavy precipitate formed and the inhibitory titre of the supernatant was reduced. If the suspension was first diluted 1 in 10 in normal saline, there was no significant change in inhibitory titre after heating at 65° C. for 30 minutes, or boiling for 2 minutes.

Action of RDE on Membrane Inhibitor.

The action of RDE on the inhibitor was tested as follows:

1 ml. of membrane suspension and 0·25 ml. RDE (1,000 units) were incubated at 37° C. Membrane suspension was incubated with normal saline as a control. The action of RDE was stopped after one hour by diluting the mixture 1 in 10 in normal saline and heating at 65° C. for 30 minutes, the control being treated similarly. The inhibitor content of each mixture was titrated. Table 2 shows the marked reduction in inhibitory titre produced by RDE.

TABLE 2.

Action of RDE on inhibitor in ground membrane suspension.

Membrane incubated with	Inhibitory titre	
	H-LEE	T-MEL
RDE (1,000 units)	<10	<10
Normal saline	480	750

Inhibitor titrated against 5 A.D. of each virus.

Presence of Intracellular Inhibitor.

Stone (1947) has shown that treatment with RDE reduces the capacity of the lining of the allantoic cavity to adsorb influenza viruses, thus preventing infection by the viruses. The virus receptors on the cell surface demonstrated by Stone's experiments would be expected to contribute to the inhibitory titre obtained with ground membranes, and surface contamination with allantoic fluid (Svedmyr, 1947) and egg-white (Lanni and Beard, 1948), both known to contain inhibitors, might also contribute. Experiments were made to eliminate these surface sources of inhibitor by careful washing in saline, followed by the use of RDE on the intact excised membrane.

TABLE 3.

Action of RDE on intact membrane.

Treatment of membrane before grinding	Inhibitory titre of ground membrane suspension	
	H-LEE	T-MEL
Incubated with RDE (4,000 units) for one hour	240	320
Nil	480	960

Inhibitor titrated against 5 A.D. of each virus.

The membrane was removed, cleared of adhering egg-white and washed thoroughly in normal saline. It was suspended in 5 ml. of a 1 in 5 dilution of RDE (4,000 units) in calcium-acetate-saline and incubated at 37° C. for one hour. The membrane was washed in citrate-saline (to reduce activity of the RDE immediately) and then ground and suspended in 1 ml. of citrate-saline. The suspension was diluted 1 in 10 in normal saline, heated at 65° C. for 30 minutes in order to inactivate the enzyme, and titrated for inhibitory activity against H-LEE and T-MEL, with suitable controls. Table 3 shows titres representative of many such experiments.

Thus, treatment of the membrane before grinding (i.e. surface treatment) with 4,000 units of RDE caused partial reduction in inhibitory titre whereas treatment with 1,000 units of RDE after grinding caused complete reduction (see Table 2). Hence some of the inhibitor is not accessible to RDE action before grinding and is presumably not present at the surface of the allantoic membrane.

Action of Viruses on Membrane Inhibitor in vitro.

A characteristic property of soluble inhibitors is the "inhibitor gradient" (Stone, 1949 b), analogous to the red cell receptor gradient (Burnet, McCrea and Stone, 1946). The inhibitory activity of certain mucoids for different indicator viruses is destroyed by active viruses to varying extents. Thus Stone showed that the inhibitory activity of ovomucin for various indicator strains was reduced by virus action in the order H-WSE, H-LEE, T-MEL; e.g. LEE virus destroyed inhibitor for H-WSE and H-LEE but not for T-MEL, whereas MEL virus destroyed inhibitor for all three. The gradient was characteristic of a particular mucoid but varied from one mucoid to another.

TABLE 4.

Action of viruses on inhibitor in ground membrane suspension.

Active virus	Inhibitory titre (per cent.)				
	T-PR8	H-WSE	T-MEL	T-BEL	H-LEE
WSE	67	33	67	84	78
PR8	<5	17	26	34	62
MEL	<5	10	<5	21	35
BEL	<5	14	7	15	30
LEE	<5	<5	<5	<6	5

Membrane suspension incubated for 18 hours with active viruses.

Residual inhibitor titrated against 5 A.D. of indicator viruses; expressed as percentage of titre, with that indicator, of membrane incubated with normal saline.

The inhibitor gradient of membrane suspension was determined by the method of Burnet (1949) as follows. Several membranes were pooled, ground and suspended in normal saline, 1 ml. of saline per membrane. 0·25 ml. of each test virus, diluted to 100 A.D., was added to 1 ml. of membrane suspension. A drop of penicillin (containing 200 units) was added, and the mixture incubated for 18 hours at 37° C., with a control containing saline instead of virus. After incubation, each mixture was diluted 1 in 10 in normal saline and heated at 65° C. for

30 minutes to inactivate virus haemagglutinin. The inhibitory content was titrated against the test viruses in the indicator state. The results of a representative experiment are shown in Table 4, where the inhibitory titres following virus action are expressed as percentages of control titres.

It is seen that the order in which inhibitors were destroyed was T-PR8, H-WSE, T-MEL, T-BEL, H-LEE. The increasing order of activity of the viruses was WSE, PR8, MEL, BEL, LEE.

Action of Viruses on Membrane Inhibitor in vivo.

Since some viruses were found to be very active enzymically on membrane inhibitor *in vitro* it was of interest to see whether membrane inhibitor was destroyed during growth of influenza virus in the developing chick embryo. The time of disappearance of inhibitor from the membrane was investigated in parallel with the time of appearance of haemagglutinin.

Henle and Henle (1949) have shown that when a large amount of PR8 virus is injected into the allantoic sac, multiplication of virus as measured by increase in haemagglutinin is first detected in the chorio-allantoic membrane after about 4 hours, and generally 1 hour later in the allantoic fluid. In the present experiments disappearance of inhibitor and production of haemagglutinin in the membrane were traced after inoculation with MEL virus, the "lag" period for MEL virus, i.e. the time before the increase in haemagglutinin in the allantoic fluid, having previously been found to be about 5 hours (unpublished experiments).

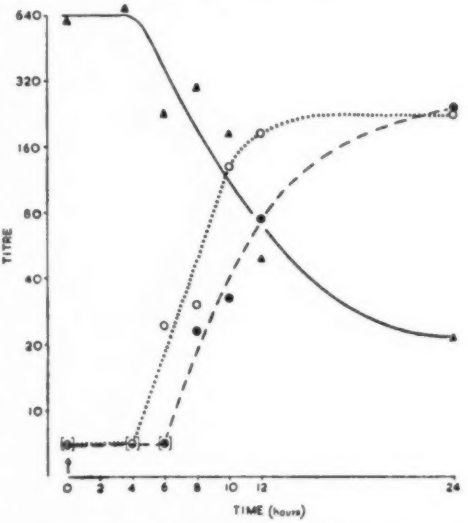

Fig. 1. Reduction of Inhibitor in Membrane during growth of MEL virus.

▲——▲ Inhibitory titre of membrane suspension against 5 A.D. of T-MEL.

O . . . . O Haemagglutinin titre of membrane suspension.

●- - - - ● Haemagglutinin titre of allantoic fluid.

The points in brackets represent titres less than the value shown.

0·1 ml. of 1 in 10 dilution of MEL (28 A.D.) was inoculated allantoically into each of a group of 21 11-day embryos which were incubated with 6 control embryos at 35° C. At 2 hourly intervals, three inoculated eggs and one control were removed from the incubator, samples of allantoic fluid taken, and the membranes removed. These were washed, ground and suspended each in 1 ml. of normal saline. A portion was immediately diluted 1 in 10, heated at 65° C. for 30 minutes to inactivate haemagglutinin, and titrated for inhibitor

against T-MEL. At the same time, the allantoic fluids and unheated membrane suspensions were titrated for haemagglutinin. Controls showed that inhibitor present in the membrane and fluid did not affect the haemagglutinin titre of MEL virus in this concentration, since this titre was not increased by treating the samples with excess RDE.

The results are plotted in Fig. 1. Each point represents the geometric mean of results of individual titrations of three samples.

Thus MEL virus caused marked reduction of the membrane inhibitory titre *in vivo*, the drop in titre beginning at the same time as haemagglutinin appeared in the membrane.

A possible objection to the technique is that virus might react with membrane inhibitor released during grinding. In a number of control experiments, in which varying amounts of virus and ground membrane were left at room temperature for periods up to an hour, there was no demonstrable drop in inhibitory titre, i.e. virus did not act appreciably on membrane inhibitor in less than an hour, at room temperature.

Membrane Inhibitor Gradient in vivo.

The inhibitor gradient *in vivo* was determined by titrating the residual inhibitor in membranes taken from infected embryos. Eggs were inoculated allantoically with 10^4 ID_{50} of virus, and after 42 hours' incubation, membranes were removed and ground. They were suspended in normal saline, diluted 1 in 10, heated at 65° C. for 30 minutes and titrated for inhibitory activity against five different indicator strains.

The results of these experiments were variable. The general picture showed that the order of the inhibitor gradient was the same as *in vitro*, i.e. T-PR8, H-WSE, T-MEL, T-BEL, and H-LEE. However, LEE and BEL viruses were not as active *in vivo* as expected from the *in vitro* results. Thus LEE virus, which was more active than MEL *in vitro*, showed a much lower action *in vivo* even when LEE infected eggs were incubated for 48 hours longer than the MEL eggs.

Allantoic Fluid Inhibitor Gradient.

The inhibitor gradient of normal allantoic fluid was investigated at the same time as that of the membrane suspension. Modification of the method used for membrane inhibitor was necessary, because long incubation of allantoic fluid with most viruses caused almost complete destruction of inhibitory activity.

1 ml. of allantoic fluid was incubated at 37° C. for one hour, with 0·25 ml. of virus containing 25 A.D. and with normal saline as a control. Haemagglutinin was inactivated by heating at 65° C. for 30 minutes and the inhibitory activity titrated.

The inhibitor gradient of allantoic fluid was found to be H-WSE, H-LEE, T-MEL. For these three viruses the gradient of membrane suspension was H-WSE, T-MEL, H-LEE, and that of ovomucin H-WSE, H-LEE, T-MEL (the latter from Stone, 1949b).

The Effect of Interference on the Action of Viruses on Membrane Inhibitor.

It was of interest to see whether the suppression of haemagglutinin production occurring in interference was accompanied by a reduction in the activity of virus on membrane inhibitor. The heated virus used as an interfering agent is enzymically inactive and would not therefore be expected to affect the experimental results.

Interference experiments were carried out as in Methods, using H-LEE as interfering virus and MEL as challenge. After the allantoic fluids were harvested and titrated, the membranes were removed from the eggs, and suspensions prepared and heated to destroy haemagglutinin. Inhibitor against T-MEL was estimated: the results of a representative experiment are shown in Table 5.

TABLE 5.

Lack of inhibitor destruction in interference.

Inoculum	Mean haemagglutinin titres of allantoic fluids	Mean inhibitory titres of chorio-allantoic membranes
Nil—control	—	480
H-LEE—control	—	420
H-LEE + active MEL	17	380
Active MEL	450	17

Interfering virus—400 A.D. of H-LEE.
Challenge virus—10^2 ID_{50} active MEL.
6 eggs per group: geometric mean titres are shown.
Inhibitor titrated against 5 A.D. of T-MEL.

It can be seen that where interference has considerably reduced haemagglutinin production no significant inhibitor destruction can be detected.

DISCUSSION.

The properties of inhibitor in chorio-allantoic membrane suspensions are similar to those described for other inhibitors of influenza virus haemagglutination: membrane inhibitor is heat stable, has greater inhibitory activity against heated than unheated virus, is sensitive to the enzymic action of influenza viruses and the receptor-destroying enzyme of *V. cholerae*, RDE, and shows a characteristic inhibitor gradient. It is interesting that quite a large part of the inhibitory activity of the membrane suspensions is not accessible to the action of RDE *before* the membranes are ground. This suggests that part of the inhibitor is intracellular—a suggestion which is supported by the fact that inhibitor is accessible to the action of influenza viruses during their multiplication *in vivo*.

The enzymic action of viruses on membrane inhibitor *in vivo* might occur during the initial stages of multiplication of virus, i.e. before haemagglutinin is produced or at a stage when the increase in haemagglutinin can be detected in the membrane. The results favour the latter alternative. Interference inhibited haemagglutinin production and inhibitor destruction, and inhibitory activity in the membrane was not reduced before increase in haemagglutinin could be detected (Fig. 1).

The inhibitor gradient of chorio-allantoic membrane suspension differs from that of allantoic fluid. This is evidence against Svedmyr's (1948) assumption that allantoic fluid inhibitor represents cellular receptor substance in solution.

During speculation on the function of intracellular inhibitor in virus multiplication, the possibility of "intracellular virus receptors" arose. de Burgh (1948) has shown that tissue particles separated from normal rat lung by differential centrifugation were agglutinated by influenza viruses, that the combination of particles and virus inhibited virus haemagglutination, and that RDE prevented combination of virus and tissue particles. Intracellular virus receptors might play a part in the interference phenomenon, particularly in view of the high affinity of heated (interfering) virus for chorio-allantoic membrane inhibitor. If so, quantitative differences in behaviour in interference might be expected among different viruses depending on their relative positions in the chorio-allantoic membrane inhibitor gradient. This possibility is at present under investigation.

SUMMARY.

Some properties of inhibitor of haemagglutination by influenza viruses found in suspensions of ground chorio-allantoic membranes have been described.

Membrane inhibitor is heat-stable, has greater inhibitory activity against heated than unheated viruses, and is destroyed by the *V. cholerae* enzyme, RDE.

In the intact membrane, part of the inhibitor is inaccessible to the action of RDE and is probably intracellular.

Membrane inhibitor and allantoic fluid inhibitor differ from one another in the order of their inhibitor gradients.

Influenza viruses act enzymically on membrane inhibitor both *in vitro* after normal membrane has been ground and *in vivo* during growth of the viruses in the developing chick embryo.

During interference, when production of virus haemagglutinin is prevented, no reduction in inhibitor can be demonstrated.

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THE LYSIS BY COMPLEMENT OF SHEEP ERYTHROCYTES SENSITIZED WITH EXTRACT OF MYCO. TUBERCULOSIS AND ANTI-BCG RABBIT SERUM

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(Accepted for publication 10th July, 1950.)

Studies on the adsorption of bacterial products to red blood corpuscles have already been reported from this laboratory. A haemagglutinin has been demonstrated in *H. pertussis* (Keogh, North, and Warburton, 1947, and Keogh and North, 1948), and the agglutination by specific antisera of erythrocytes coated with bacterial polysaccharides has been described (Keogh, North, and Warburton, 1947 and 1948, and Warburton, Keogh and Williams, 1949).

Middlebrook and Dubos (1948) using similar methods obtained extracts of *Myco. tuberculosis* which were capable of sensitizing sheep erythrocytes to agglutination by specific antisera.

We had no difficulty in reproducing the results of Middlebrook and Dubos (1948) with rabbit immune sera; in tests on sera of healthy and infected cattle, however, irregular and non-specific results were obtained. Pound (1949) has observed similar anomalous agglutinations with bovine sera.

In an experiment set up to test for agglutination of coated cells by the serum of a tuberculous guinea-pig, lysis of the cells was observed; the serum was fresh and had not been inactivated. This observation suggested that sensitized cells were haemolyzed in the simultaneous presence of antibody and complement. This hypothesis was borne out by subsequent experiments.

The phenomenon has been reported in a preliminary note (Fisher and Keogh, 1950). A more detailed account of the work done with rabbit serum is given below. Results obtained with bovine sera will be reported later.

Materials and Methods.

Preparation of sensitizing extracts. 8 gm. heat-killed, dried organisms of the virulent human type PN strain were agitated in 200 ml. 90 p.c. phenol in a Waring blendor until all lumps had disintegrated; the temperature was not allowed to rise over 40° C. The supernatant was separated on the centrifuge and discarded. The jelly-like insoluble material was subjected to repeated extractions with 90 p.c. phenol, until a sample of the separated supernatant gave no precipitate when poured into 3 volumes of alcohol. The phenol-insoluble residue was washed three times with 200 ml. lots of acetone, dried in the incubator and stored at room temperature. Aliquots were extracted, as required, with the solution (0·55 p.c. NaCl, 0·5 p.c. Na_2HPO_4 and 20 p.c. methanol in water) used by Middlebrook and Dubos (1948). One gm. phenol-insoluble

material was suspended in 50 ml. solvent by the aid of pestle and mortar, and the suspension placed in a flask on the shaking table in the 37° C. room overnight. Filtration of the suspension through paper on a Buchner funnel yielded a faintly opalescent, slightly greenish solution.

Sensitization of red cells. Sheep were bled aseptically from the vein into modified Alsever's solution (Bukantz, Rein and Kent, 1946) and the blood preserved in the refrigerator for periods up to a fortnight. The cells were washed three times in saline immediately prior to use.

One volume of packed washed cells was suspended in 10 to 20 volumes of sensitizing extract. The latter is isotonic with respect to crystalloids, and the presence of the methanol in no way seemed to affect the erythrocytes. The suspension was allowed to stand on the bench for 20–30 minutes, with occasional shaking, then the cells were centrifuged out, washed twice in saline and resuspended in saline to a final concentration of 5 p.c.

Sera. A reference antiserum was prepared in rabbits against BCG organisms according to the method of Middlebrook and Dubos (1948). The serum was inactivated at 56° C. for 30 minutes and stored in the cold room with 0·01 p.c. merthiolate as a preservative.

Complement. Guinea-pig serum, not more than 24 hours old, was used.

Absorption of serum and complement. This step was included in order to remove antibodies to sheep erythrocytes. The complement was diluted with an equal volume of saline, and the serum to the desired degree. Packed, washed sheep erythrocytes, equal in volume to the amount of undiluted serum or complement, were then mixed with the dilutions. The serum was absorbed on the bench for 15–20 minutes, and the complement in an ice-water bath for the same length of time. The absorbing cells were then removed by the centrifuge.

Agglutination and haemolytic tests. Twofold serial dilutions of the serum were prepared in 10 drop volumes in Wassermann tubes. One drop of the 5 p.c. suspension of the sensitized cells was added to each tube. The following controls were set up: normal (unsensitized) sheep cells in the first serum dilution, and normal and sensitized cells in saline. The suspensions were thoroughly mixed and placed in the incubator for at least forty-five minutes; the tubes were then read for agglutination. One drop of absorbed 1 in 2 complement was then added to each tube, giving a final dilution of complement of 1:24. The contents were mixed and the racks placed in the 37° C. water-bath for 45 minutes. The tubes were then read for haemolysis.

Titres are expressed as the reciprocal of the highest dilution of the serum giving complete agglutination or haemolysis.

RESULTS.

Portions of the same lot of sensitizing extract were used for these tests.

Number of Sensitizing Doses present in Extract.

Doubling dilutions of the extract were prepared in the methanol-phosphate-saline of Middlebrook and Dubos (1948) and one drop of packed cells sensitized with 20 drops of each dilution. The coated cells were tested for agglutinability by dilutions of the reference antiserum, and for haemolysis after the addition of complement.

The results (Table 1) indicate that:

(i) just under 16 minimal complete sensitizing doses were present in the extract,

(ii) the susceptibility to haemolysis was, in terms of serum dilution, much greater than the susceptibility to agglutination, and

(iii) with increasing dilution of the sensitizing agent, i.e. with decreasing degree of coating of erythrocytes, more serum was required for agglutination and haemolysis; the ratio of agglutinating to haemolytic titre, however, remains constant over the whole range.

TABLE 1.

Number of sensitizing doses present in extract.

Dilution of Sensitizer	Tested for	Dilution of serum								Saline controls
		1/20	1/40	1/80	1/160	1/320	1/640	1/1,280	1/2,560	
1	A*	+	+	Tr	—	—	—	—	—	—
	H†	+	+	+	+	+	+	+	±	—
1/2	A	+	+	Tr	—	—	—	—	—	—
	H	+	+	+	+	+	+	±	±	—
1/4	A	+	+	Tr	—	—	—	—	—	—
	H	+	+	+	+	+	+	±	±	—
1/8	A	+	+	Tr	—	—	—	—	—	—
	H	+	+	+	+	+	+	±	±	—
1/16	A	+	+	Tr	—	—	—	—	—	—
	H	+	+	+	+	+	+	±	Tr	—
1/32	A	±	Tr	—	—	—	—	—	—	—
	H	+	+	+	+	+	±	Tr	—	—
1/64	A	—	—	—	—	—	—	—	—	—
	H	+	+	+	+	±	±	Tr	—	—
1/128	A	—	—	—	—	—	—	—	—	—
	H	+	+	+	±	±	Tr	—	—	—
1/256	A	—	—	—	—	—	—	—	—	—
	H	+	±	±	Tr	—	—	—	—	—
1/512	A	—	—	—	—	—	—	—	—	—
	H	Tr	Tr	—	—	—	—	—	—	—
1/1024	A	—	—	—	—	—	—	—	—	—
	H	—	—	—	—	—	—	—	—	—
Controls (normal cells)	A	—	—	—	—	—	—	—	—	—
	H	—	—	—	—	—	—	—	—	—

* A = agglutination.
† H = haemolysis.
\+ = complete agglutination or haemolysis respectively.
± = partial agglutination or haemolysis respectively.
Tr = trace of agglutination or haemolysis respectively.
— = no agglutination or haemolysis respectively.

Activity in Terms of Dry Weight.

5 ml. of extract were adjusted to pH 5·5 with N/10 HCl, and 15 ml. ethanol added, after having cooled both reagents to between —5° and —10° C. The extract was acidified in order to avoid precipitation of the phosphates by the alcohol. After a few minutes' standing, the mixture was separated on the refrigerated centrifuge and the supernate set aside in the cold. The precipitate was washed once, at the same temperature, with a solution consisting of

methanol (1 part), distilled water (4 parts) and ethanol (15 parts), and again centrifuged. The washings were discarded and the precipitate dissolved in 5 ml. distilled water (Fraction 1). The supernate was precipitated in the cold with a further lot of 35 ml. ethanol, and the separated precipitate dissolved in 5 ml. distilled water (Fraction 2).

Portions of the two fractions were rendered isotonic by the addition of appropriate amounts of a 30 p.c. solution of NaCl in water, and tested for their ability to sensitize erythrocytes to haemolysis. Fraction 1 contained approximately 75 p.c. of the activity of the original extract. None was found in Fraction 2.

1 ml. of Fraction 1 was evaporated to dryness and the last traces of moisture driven off at 104° C. The residue weighed 1·5 mg. The fluid extract therefore contained not more than 0·15 p.c. of active material.

Assuming that all the active material was recovered in Fraction 1, and that loss of 25 p.c. of the activity was due to denaturation, the minimal detectable sensitizing concentration of the coating agent was of the order of 1 part in 300,000 since, under the experimental conditions, cells coated with a 1:512 dilution still showed a trace of haemolysis (Table 1).

Effect of Varying the Amounts of Antiserum and Complement.

The cells which had been sensitized with twenty volumes of extract were suspended in rows of tubes containing dilutions of the reference antiserum. Undiluted complement was absorbed with normal sheep cells in the usual way and then diluted serially twofold in saline. One drop of each dilution of complement was added to each of a row of tubes containing the graded dilutions of the reference antiserum and the sensitized cells.

The results are shown in Table 2. With decreasing amounts of complement, more antiserum is required to haemolyze a given quantity of cells, and vice versa; i.e. the haemolytic titre of the serum is higher if more complement is used.

TABLE 2.

Haemolysis of sensitized cells in the presence of varying amounts of serum and complement.

Final complement dilution	Serum dilution								Controls (no serum)
	1/20	1/40	1/80	1/160	1/320	1/640	1/1,280	1/2,560	
1/12	+	+	+	+	+	+	+	+	—
1/24	+	+	+	+	+	+	+	±	—
1/48	+	+	+	+	+	+	±	±	—
1/96	+	+	+	+	+	+	±	—	—
1/192	+	+	+	+	+	±	Tr	—	—
1/384	+	±	±	±	±	Tr	—	—	—
1/768	—	—	—	—	—	—	—	—	—
Controls (no complement)	—	—	—	—	—	—	—	—	—
Normal cells, 1/20 serum, 1/12 complement	—								

+ = complete haemolysis. ± = partial haemolysis. Tr = trace of haemolysis.
— = no haemolysis.

Effect of Heating the Complement.

Complement which had been heated to 55° C. for 30 minutes was no longer capable of causing haemolysis.

Test with Normal Rabbit Serum.

No haemolysis occurred in systems containing normal rabbit serum, sensitized sheep red cells and complement.

Neutralization of Serum Haemolysis by Sensitizing Extract.

One drop of a 1 in 256 saline dilution of sensitizing extract was added to each of a row of tubes containing 10 drops of dilutions of the reference serum. One drop of saline was added to the tubes of a control row. The mixtures were incubated at 37° C. for 30 minutes, sensitized cells added, and, after further incubation, complement, and haemolysis allowed to take place.

It was found that addition of the sensitizing extract reduced the haemolytic titre of the serum to one quarter of the original titre. The possibility that this reduction was due to fixation of the complement by antigen-antibody complex in the bulk phase of the system was excluded by the following experiment:

Extract and antiserum in amounts equivalent to, or larger than, those used in the above experiment, were incubated together and complement added. After further incubation, the amount of free complement was estimated by testing serial dilutions against sheep cells sensitized with amboceptor, using equivalent complement dilutions in saline as controls. Between 8 and 16 minimal haemolytic doses were present in the controls. The amount of complement fixed in the mixtures containing the largest amounts of extract and antiserum was less than one minimal haemolytic dose.

DISCUSSION.

Middlebrook and Dubos (1948) reported that sheep erythrocytes, when coated with an extract of the phenol-insoluble residue of tubercle bacilli, became agglutinable by specific antisera. Their findings have been confirmed. Furthermore, it has been shown that haemolysis occurs when guinea-pig complement is added to the suspension of sensitized cells in immune serum.

The amounts of antibody and complement necessary to cause lysis of a given quantity of erythrocytes are reciprocally interdependent in the conventional sheep cell-amboceptor-complement haemolytic system (Noguchi, 1923; Hyde and Parsons, 1927) as well as in the haemolytic system described above. Moreover, in both systems, more antibody is required to agglutinate a specified quantity of the appropriate cells than is necessary for lysis in the presence of excess of complement. Sheep erythrocytes sensitized with extract of *Myco. tuberculosis*, therefore behave, in some respects, in the same way in the presence of anti-BCG rabbit serum and complement as do normal sheep cells in the presence of amboceptor and complement.

Leucocytes undergo lysis at an increased rate in the presence of tuberculin, immune serum and complement (Favour, 1949; Miller, Favour, Wilson and Umbarger, 1949 (a) and (b); and Miller, Vaughan and Favour, 1949). The mechanism of this phenomenon and of the one described above may be similar.

SUMMARY.

Sheep erythrocytes, coated with an extract of the phenol insoluble residue of tubercle bacilli, and suspended in dilutions of the inactivated serum of a rabbit immunized with BCG, are haemolyzed in the presence of guinea-pig complement.

Addition of the sensitizing extract to the serum is capable of neutralizing the haemolytic antibody.

Some aspects of the above phenomena have been subjected to quantitative investigation.

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EFFECT OF SODIUM HYDROXIDE ON THE MELANOPHORE-EXPANDING PROPERTIES OF PITUITARY EXTRACTS

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(Accepted for publication 27th May, 1950.)

Injection of extracts of anterior lobe, neuro-intermediate lobe or whole pituitary into pale amphibia evokes temporary expansion of melanophores and darkening of skin. The melanophore excitant, "B", is located in the intermediate lobe of most vertebrates; in those without a morphologically distinct intermediate lobe it is manufactured in the anterior lobe. "B" is a true hormone in those animals that exhibit visible colour change in response to different visual stimuli (Waring, 1942). The pharmacological properties of "B"—containing extracts and their biological assay have been described previously (Landgrebe *et al.* 1941, 1943, 1944), and are summarized by Waring and Landgrebe (1950).

The melanophore excitant properties of extracts from different glands have been described previously (Waring and Landgrebe, 1949).

Extracts prepared in different ways from the *same original* pituitary powder may differ with regard to degree and duration of their effect on melanophores. The present paper presents an analysis of these differences.

METHODS.

Measurement of Potency and Duration of Response.

Except where otherwise stated, the melanophore response of *Xenopus* was used for assay of "B" activity in extracts (Landgrebe and Waring, 1944, 1950). Potency of an extract is expressed in "International Units" and is a measure of the degree of melanophore expansion (increase of melanophore index) attained and takes no account of the duration of response.

After caustic soda treatment (p. 0) extracts commonly exhibit increased potency and/or increased duration of response. Increase of potency we call *potentiation*; increased duration of response we call *protection* because indirect evidence implies that it is due to reduced rate of destruction of the activity in the body. Fig. 1 shows how degrees of *potentiation* and *protection* can be expressed.

Degree of *potentiation* can be expressed as the ratio of the actual *increase* in potency of an extract after treatment compared with its potency before treatment.

i.e. $\text{Potentiation} = \dfrac{\text{Potency treated} - \text{Potency untreated}}{\text{Potency untreated}}$, and since potency is the inverse of dose, in this example it equals 1·1. On this notation, no potentiation = *0*.

Degree of *protection* can be expressed as the ratio of the *increase* in time necessary for the melanophores to move from m.i. 3·0 to m.i. 2·0 when injected with treated extract as compared with time to make the same transition after injection with the same extract untreated in dose sufficient to attain the same peak m.i. So if t = the time taken for the m.i. to fall from 3·0 to 2·0 after injection of untreated extract, and t_1 is the time taken for the m.i. of the same toads to make the same transition (3·0 → 2·0) after injection of the treated extract, then

Protection $= \frac{t_1 - t}{t}$ i.e. in the above example = 0·83. On this notation no protection = *O*.

Theoretical Considerations.

No satisfactory method has yet been evolved for accurate estimation of the hormone circulating in normal animals, and it is therefore important to consider what the melanophore response curve tells us of the blood concentration.

When an acid extract of the posterior lobe is injected into a pale *Xenopus*, its melanophores slowly expand and reach a maximum expansion in about 1½ hours irrespective of dose. With doses evoking submaximal responses, the melanophores then slowly contract. Perfusion experiments (Waring and Landgrebe, 1941) show that *Xenopus* melanophores take about 1½ hours to expand fully from m.i. = 1 to m.i. = 5 and that the speed is not greatly affected by dose. So, after injection, effector speed is probably the limiting factor in the time taken for the animal to darken. Consequently the gradual rise of index after injection tells us little about the changing concentration of hormone in the blood except that an effective concentration is obtained quickly. Perfusion experiments also show that fully expanded melanophores can contract in one hour. Injected toads always take longer than an hour to resume pallor after peak expansion. So for this part of the melanophore response curve, effector time is not the limiting factor. It appears, therefore, that some hormone is still in circulation until just before pallor is regained.

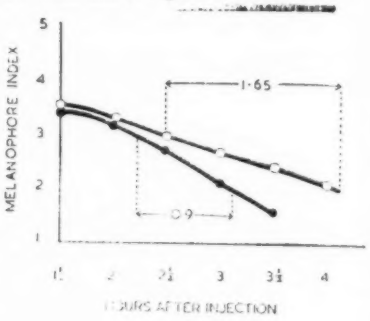

Fig. 1.

• Average m.i. 12 *Xenopus* injected with standard extract of International Standard Powder. Diluted 1 in 120 and 0·2 ml. injected into the dorsal lymph sac, i.e. amount injected = 3·3 milliunits.

° Average m.i. same 12 *Xenopus* injected with same extract treated. Diluted 1 in 250 and 0·2 ml. injected into dorsal lymph sac, i.e. amount injected = 1·6 milliunits which gives the same peak m.i.

"Treatment" = extract adjusted to pH 13 and put into boiling water bath for 2 minutes, then neutralized.

It would appear from this that the slope of the response curve after the peak is passed gives some index of the amount of "B" still circulating. Since this slope is the same for widely differing doses of an acid extract it appears that the rate of removal of "B" is constant. This rate of removal cannot, however, be deduced from existing experimental evidence and must await work on three things:

(1) Actual estimation of blood concentration of "B"; (2) Relative sensitivity of melanophores at different levels of expansion; (3) How long the hormone is effective at the melanophore surface after it has been withdrawn from general circulation.

What we do know, however, is that animals with few melanophores need less "B" hormone to activate them than do animals with abundant melanophores (Landgrebe and Waring, 1944).

Potentiation after caustic soda treatment may be due to activation of a precursor or to modification of the "B" molecule resulting in a more potent derivative (Landgrebe and

Waring, 1941/43). Previously we suggested a convenient notation which avoids periphrasis without prejudging the issue of exactly what happens during caustic soda treatment, viz. "B" is the excitant presumed to be present in the gland; "B_1" is the additional activity (as measured by peak m.i. and not duration of response) resulting from caustic soda treatment. *Protection* is probably due either to alteration of the "B" molecule which results in delayed excretion or destruction, or to a different qualitative effect of the excitant on the melanophore itself.

Since different groups of toads take different times for the melanophore index to return from m.i. 3 to m.i. 2, and give different maxima, both the indices of protection and potentiation must be worked out after injection of the same group of toads with both treated and untreated extracts.

EXPERIMENTAL OBSERVATIONS.

Effect of Temperature and pH on the Properties of "B" Containing Extracts.

Hogben and Gordon (1930) first showed that caustic soda treatment of a pituitary extract, besides destroying its pressor properties, also increased its melanophore expanding powers. They used strong caustic soda (1·35N) in the cold for two hours and made no reference to an increased duration of response. More recently it has been customary to adopt a more rapid procedure which also destroys the pressor property and increases the melanophore expanding potency of dilute extract, viz., adding one-tenth of the volume of N.NaOH and heating it in a boiling water bath for ten minutes. This method, however, gives erratic results, and the effect of heating the extract for different times at different pH values was investigated.

TABLE 1.

Potentiation and protection at different pH values

Standard extract of International Standard Powder 0·1 ml. + 0·9 ml. buffer (glycine) adjusted to pH as noted (with cation correction) on Marconi glass electrode meter, followed by *2 minutes* in B.W.B., and neutralized.

pH	Potentiation	Protection
8·0–12·0	0	0
12·5	0·3	0·5
12·6	0·5	0·5
12·8	1·0	0·7
13·0	1·0	0·8

No loss or gain of activity was detectable after B.W.B. treatment within the pH range 8–12.

Tables 1 and 2 summarize our findings which show why results are erratic when heat treatment is continued for approximately ten minutes and the pH is not accurately adjusted.

We now heat dilute extracts for two minutes at pH 13·0 in a B.W.B. and this treatment gives consistent results. Table 3 shows:

(a) *potentiation* and *protection* do not always parallel one another.

(b) caustic soda treatment of pituitaries from frogs equilibrated in darkness does not evoke greater *potentiation* and/or *protection* than does similar treatment of pituitaries from frogs equilibrated in light. This is contrary to the claim advanced by Koller and Rodewald (1933), and reinforces evidence of a similar kind obtained with dogfish glands (Waring *et al.*, 1942). The figures presented in Table 3 indicate the possibility that the *protection* of caustic-soda-treated extracts from frog glands kept in darkness is slightly greater than that of similarly treated animals kept in light, but the difference is probably not significant in view of the small number of animals used.

(c) L.R.W.$_2$ is a "B" containing extract that has not been treated with alkali during its preparation. In its final form it is neither potentiated nor protected after caustic soda treatment. This confirms our previous results (Landgrebe *et al.*, 1943).

TABLE 2.

Effect of heating a standard extract of International Standard Powder for different times at pH 13 (pH adjusted with N. caustic soda).

Duration of treatment in minutes	Percentage oxytocic activity remaining	Potentiation	Protection
1	30	1·0	0·8
2	5	1·0	0·8
3	1	0·8	0·8
7	0	0·5	0·75
10	0	—0·2*	0·6

* i.e. slight loss of potency.

TABLE 3.

Effect of caustic soda treatment on a selection of original glandular materials.

0·25 p.c. acetic acid extract adjusted to pH 13 with N.NaOH; B.W.B. 2 minutes; neutralized N.HCl.

Extract	Potentiation	Protection
Ox—International Standard Powder (P.L.P.)	1·1	0·8
Ox—Commercial (P.L.P.)	1·0	0·8
Ox—Commercial (A.L.P.)	0·2	1·6
Dogfish (P.L.P.)	2·2	0·8
Frog* (Whole Gland—white background)	6·4	2·0
Frog* (Whole Gland—back background)	8·0	1·8
Frog* (Whole Gland—complete darkness)	7·0	2·5
L.R.W.$_2$	0†	0

* Equilibrated on these backgrounds for five weeks.

† We recorded 20 p.c. loss (Landgrebe, Reid, Waring, 1943) after 10 minutes B.W.B.

Those findings seem to dispose effectively of the idea that *potentiation* and *protection* are both due to one modification of the hormone molecule. This being so, it appeared likely that controlled changes of temperature, length of B.W.B. treatment, pH and concentration might enable either *potentiation* or *protection* to be obtained without the other.

We have tried variation of pH while keeping the other variables constant. Standard extracts of International Standard Powder were buffered at various pH values subjected to two minutes B.W.B., neutralized and assayed. The results are shown in Table 1. There is a suggestion that between pH 12–13 *potentiation* and *protection* are not exhibited equally, but no clear cut separation was obtained. Chen and Geiling (1943) made similar experiments with quite different results. This is not surprising in view of their method of assay which utilises the time for the melanophores to expand from, and to contract back to, their original condition as the criteria for comparing the potency of extracts. This is open to serious objections since it has been shown that constituents of the extract other than "B" activity affect the time of recovery of the melanophores.

Since extracts are usually sterilized for storage by immersion in a B.W.B. it seems relevant in this section to append a note on keeping qualities of various "B" containing extracts. Standard extracts were dealt with in a previous paper (Waring and Landgrebe, 1949). We can now report on two other extracts, L.R.W.$_1$ and L.R.W.$_2$ (Landgrebe *et al.*, 1943). Ampoules of L.R.W.$_1$ sterilized by B.W.B. or autoclaving at pH 3–7, and kept at room temperature in the *dark* retain their activity undiminished for at least 12 months. L.R.W.$_2$ has very erratic keeping qualities and we are unable to prescribe a method of keeping it except in the solid.

Further Analysis of Potentiation and Protection.

It is firmly established that "B" is effective on melanophores in isolated perfused limbs and in isolated pieces of skin, and it seems safe to assume for the present that it acts directly on the melanophore itself. There are three sources yielding information bearing on the further analysis of *potentiation* and *protection*.

Intact Xenopus.

During routine assays of "B" using the same test animals for some years, we gained the impression that the previous background history of the animal affected the speed of reconstraction of the melanophores after peak expansion had been reached. We also noted in some cases that animals kept on a white background for a long period exhibited *protection* but not *potentiation* after injection of treated extracts.

Direct experiment has confirmed that long sojourn on a white background increases the speed of initial melanophore expansion and the speed of recovery, irrespective of whether "B" or "B_1" is involved, and indicated that all animals exhibit *protection*. All recently-white-adapted animals exhibit *potentiation* as well, *but animals equilibrated on a white background for two years exhibit no potentiation*. It appears therefore that we must be prepared to envisage in such animals the formation of some substance in tissues or circulation which inhibits "B_1" preferentially, and "B" only to a small extent (Landgrebe and Waring, 1941).

Perfusion Experiments.

In previous perfusion experiments using *Xenopus* (Waring and Landgrebe, 1941) it was noted that when perfusion with pituitary-free saline was begun with the melanophores expanded, the melanophores slowly contracted. When, however, the melanophores had again expanded under the influence of a "B"-containing saline, subsequent perfusion with pituitary-free saline did not lead to contraction. The same was true when perfusion was started with a pale animal and a "B"-containing extract. In all these experiments a caustic-soda-treated extract was used and the inability of melanophores to contract was perhaps a clue to the seat of action of the "protection" phenomena. Consequently we reinvestigated the matter using

treated and untreated extract. Frogs (*R. temporaria*) were used in all these experiments. The animal was pithed, the dorsal aorta cannulated, the anterior abdominal, femoral, sciatic and pelvic veins cut; and the leg web placed under a 2/3 in. objective for constant observation. Perfusion fluids were run from burettes into a constant pressure cannula which was connected with the aortic cannula. Ringer's saline (buffered with phosphate and bicarbonate) was perfused until the melanophores were fully contracted and most of the blood corpuscles were removed, then saline containing posterior lobe pituitary extract *not* treated with caustic soda was perfused. We aimed at a dose which was just sufficient to cause melanophore expansion (about 0·02 "I.U."/100 ml. perfusate). When the m.i. reached 3·0, "B"-free saline was perfused. In most instances the m.i. continued to rise to 3·5 and stayed at this figure however long perfusion was continued. In others the melanophore index remained permanently at 3·0. So the permanent nature of expansion after perfusion with "B" containing extract in the original experiments was not due simply to the caustic soda treatment of the extracts employed.

Of the possible explanations for the observation that melanophores expanded by endogenous pituitary secretion contract when perfused with "B"-free saline whereas those expanded by perfusion with "B"-containing saline do not, two are easily susceptible of test: (i) all perfused specimens exhibited oedema after about thirty minutes, so the possibility existed that sufficient "B" was "locked" in the tissues to prevent melanophore contraction. We found that saline containing 1 p.c. gelatine prevented oedema, but gave similar results; (ii) no evidence exists to show whether or not acetone dehydration of ox pituitaries and the subsequent extraction of the active principle with dilute acetic alters "B" in any way. So frog pituitaries were crushed in saline and substituted for the usual extract. This gave the same results as previously. Since no information relevant to the issue under investigation seemed likely to accrue from perfusion experiments, they were discontinued.

Isolated Skin.

The effect of extracts was tried on isolated skin from dogfish (*Scyllium canicula*) and from frogs. The former has many advantages for this type of work and experiments with it will be fully reported on elsewhere. Here we may note the results relevant to our theme. *Protection as well as potentiation, is exhibited in isolated skin.* Whether the *protection*, exhibited here, is the same phenomenon as that described in intact animals is open to question, but it does lend itself more easily to further analysis. Two exigencies have to be considered:

(a) are melanophores which retain a fixed m.i. of approximately 3·0 for several hours still capable of further contraction, and

(b) if so, since their persistent and constant partial expansion must be due to persistent pituitary activity, is the melanophore expanding power of the extract diminished?

The first is readily tested by immersing the skin with expanded melanophores into saline alone. This was done several times, from different degrees of expansion evoked by both treated and untreated extract. Contraction followed in each case. We could detect no certain difference between skin from treated and untreated extract in the speed of contraction.

The second point was tested by immersing a second piece of skin into saline containing untreated extract which had already raised the m.i. of a piece of skin to 3·0. No expansion of the melanophores of the second piece of skin resulted, though those of the first piece of skin contined to expand to m.i. 4 over a period of two hours.

Under identical conditions, pieces of skin were placed in a dose of treated extract, estimated (by trial) to raise the m.i. to 3·5. As the peak (3·5) was attained a new piece of skin was inserted into the extract. The melanophores of the new piece showed very slight expansion (m.i. = 1·3), but the melanophores of the original six pieces of skin remained at m.i. = 3·5 for two hours further. Saline was then substituted for extract and the melano-

phores promptly contracted. This behaviour was confirmed in other experiments. Clearly this attack promises much useful data, and so far one conclusion is justified: *When melanophores reach a submaximal peak in a treated extract the extract is not capable of appreciably raising the m.i. in a fresh piece of skin but will maintain the melanophores in the original skin at m.i. 3·5 for a number of hours.* Two interpretations seem possible from this:

(a) that less pituitary excitant is needed to maintain melanophores at 3·5 than to raise them from 1 to 3·5, and that this minimal amount still persists constantly in *treated* but not in *untreated* extracts, or

(b) that some component of the treated extract remains adherent to or in the skin or melanophores and inhibits contractions.

SUMMARY.

Caustic soda treatment of posterior lobe pituitary extract has two effects on its melanophore excitant properties: (a) an increased potency as judged by the peak m.i. attained after injection (*potentiation*); (b) an increased duration of response (*protection*). Notations for expressing degrees of (a) and (b) quantitatively are put forward.

Evidence is adduced to show that these two effects are not due to a single alteration of the excitant. The chief evidence is: (a) heat treatment at different pH values and for different periods shows that the two effects do not run parallel (Tables 1, 2 and 3). (b) All *Xenopus* exhibiting the white background response (contracted melanophores) exhibit *protection*. Toads equilibrated for a short time also show *potentiation*, those equilibrated for long periods do not.

Attempts to elucidate further the mechanism of *protection* and *potentiation* by perfusion techniques have proved abortive.

Melanophores in isolated skin exhibit *protection* and *potentiation*, so it appears that both can be peripheral phenomena. Analysis of data from isolated skin experiments shows that *under these circumstances, protection* is due either to (a) prolonged persistence of the excitant in quantities insufficient to *raise* the m.i., but sufficient to maintain it or (b) that treated extract adheres to the melanophore at times when it is impossible to measure it in the general saline.

Acknowledgments. For expenses grants H.W. is indebted to Medical Research Council (Great Britain), Carnegie Trust (Scotland), Royal Society (London) and the Australian Commonwealth Research Grants.

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THE EFFECT OF SMALL pH CHANGES ON THE COLORIMETRIC ESTIMATION OF CARBONIC ANHYDRASE ACTIVITY—PARTICULARLY THOSE CHANGES CAUSED BY ZINC IONS

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(Accepted for publication 19th June, 1950.)

Roughton and Booth (1946) have introduced a method of assay of carbonic anhydrase whereby the difference in the time taken for saturated CO_2 to reduce the pH of veronal buffer from 7·9 to 6·4 in the presence of enzyme compared with a blank with no enzyme, is taken as an index of activity. Using this method slightly modified (as described by Trethewie and Day, 1949) the effect of metallic ions, particularly zinc, was noted on the $CO_2 + H_2O \rightarrow H_2CO_3$ reaction. Zinc showed inherent activity when this method of assay was used, and in view of the presence of zinc in the carbonic anhydrase enzyme complex (Keilin and Mann, 1939), it was thought worthwhile to investigate the rôle this metal played in altering the reaction time. In the following work the effect of zinc was investigated and it was attempted to establish the reason for the apparent changes in activity demonstrated by this test when small amounts of zinc were present in the buffer.

Method.

Carbonic anhydrase activity was assayed by means of the method of Roughton and Booth (1946), as modified and used by Trethewie and Day (1949). In most experiments no source of carbonic anhydrase was used, the blank test being compared with the blank test plus the metal in the required concentration. The "activity" of the metal in various concentrations was expressed as the number of seconds difference between the blank and the test solution.

The pH determinations were carried out by means of a Cambridge electric pH meter.

The blood used was rat blood drawn from the heart immediately after death, centrifuged and the cells diluted appropriately with glass distilled water to 100 parts per million (p.p.m) for use as a source of carbonic anhydrase.

The buffer used was 0·02 M veronal buffer prepared by dissolving 3·680 gm. Barbitone (diethylbarbituric acid) and 4·120 gm. Soluble Barbitone (sodium diethyl barbiturate) in 1 litre of glass distilled water. The pH was determined as 8·2 in the case of the first buffer and 7·88 for the second buffer used. 0·01 M buffer was simply prepared by diluting the 0·02 M with an equal quantity of glass distilled water. Borate buffer was prepared by dissolving 3·10 gm. boric acid, 3·73 gm. potassium chloride and 0·340 gm. of sodium hydroxide in 500 c.c. of glass distilled water (pH = 8·25).

EXPERIMENTAL.

The Apparent carbonic anhydrase enzyme activity of zinc in veronal buffer.

To stoppered weighing bottles kept at 0° C. in ice and water were added 3 ml. of veronal buffer (pH = 8·2), 2 ml. of glass distilled water and 3 drops of bromothymol blue. To this, 5 ml. of saturated CO_2 at 0° C. were added and the time was taken for the pH to fall to 6·4. This was observed colorimetrically by comparison with a phosphate buffer at this pH. In another series of weighing bottles a similar test was made, only instead of 2 ml. of glass distilled water, 1 ml. only was used plus 1 ml. of 1/1,000 Zn++ as zinc sulphate, thus giving a final Zn++ concentration of 1/10,000. These tests, together with the blanks described above, were randomized and the "activity" determined. The average activity of the blank was 72·8 seconds, whereas that of the zinc (1/10,000) was 52·9 seconds (average of three tests), showing a quickening of the reaction or an "activity" of 19·9 seconds. On another day an identical series was carried out, this time the average blank being 73·9 seconds and the zinc 53·0 seconds, showing an "activity" of 20·9 seconds. When the concentration of zinc is reduced the "activity" which it exerts is reduced accordingly, but even in small amounts significant differences in time are present when the zinc preparations are compared with the blank tests. When 1 ml. of 1/10,000 Zn++ is added (i.e. making a final concentration of 1/100,000) the average difference between the blank and the zinc tests were in various series, as follows: 2·0, 5·5, 2·3, 4·4 and 1·7 seconds respectively, showing that even in as low a concentration as 1/100,000 a significant decrease in time is shown when zinc ions are added to the test solution. The results of these tests with additional data are shown in Table 1. In all cases the averages shown in the various columns are the result of a series of tests carried out on the same day.

TABLE 1.

The effect of zinc on the reaction time.

Date	Blank (Av.) (seconds)	Final zinc concentration	Test (Av.) (seconds)	Activity	pH buffer
14/10/49	72·8	1/10,000	52·9	19·9	8·2
17/10/49	79·2	1/100,000	77·2	2·0	8·2
17/10/49	73·0	1/100,000	67·5	5·5	8·2
18/10/49	72·8	1/100,000	70·5	2·3	8·2
18/10/49	72·8	1/100,000	68·4	4·4	8·2
20/10/49	72·0	1/100,000	70·3	1·7	8·2
7/11/49	73·9	1/10,000	53·0	20·9	7·88

It is seen from these results that zinc in small amounts decreases the time required for CO_2 to reduce the pH of veronal buffer to 6·4. The difference in low concentrations of zinc was small, admittedly, but when subjected to statistical analysis proved significant ($t = 4·5$ $p < ·001$). It seemed possible that zinc and veronal formed a complex with enzymic activity and, if this were so, interesting conclusions could be drawn since carbonic anhydrase itself is the only known compound of zinc with the enzyme activity.

However, subsequent investigations described here show that the action of the zinc can be explained satisfactorily without attributing to it any enzyme activity. In view of this fact, the possibility of a zinc-veronal complex loses its significance, but it is of interest, however, to note various facts which point in this direction, and work was commenced to establish the presence of a complex.

Four solutions were prepared. In the first (Solution I) to 30 ml. of veronal buffer (0·02 M) were added 10 ml. of 1/1,000 Zn++ and 30 ml. of glass distilled water. No precipitate resulted and the pH was found to be 7·70. The second solution (Solution II) contained 30 ml. of veronal buffer, to which were added 10 ml. of 1/1,000 Zn++ and then 30 ml. of borate buffer, with no resulting precipitate. The pH was found to be 8·08. The third solution (Solution III) contained 15 ml. of borate buffer, 5 ml. of 1/1,000 Zn++ and 15 ml. of glass distilled water. The pH was 8·09 and the zinc was precipitated. The fourth solution (Solution IV) contained 15 ml. of borate buffer and 5 ml. of 1/1,000 Zn++ and as soon as the precipitate appeared, 15 ml. of veronal buffer were added with the complete disappearance of the precipitate. The pH was 8·06.

We see that when zinc sulphate solutions are placed in borate buffer (pH 8·25), an immediate precipitate results ($Zn(OH)_2$). One would expect such a precipitate with veronal buffer of similar pH since these buffers represent H+ concentrations on the alkaline side of neutrality. Further, the addition of veronal buffer to $Zn(OH)_2$ precipitated in borate buffer results in a disappearance of the precipitate. It can be seen also that Solutions II, III and IV have almost identical pH. The only difference between those in which there is no precipitate and those in which a precipitate appears is the presence of the veronal. These facts suggest that the zinc ions are combined in some way with veronal buffer. Unfortunately the final step in establishing the presence of any such complex could not be taken since an ultra-violet spectrophotometer was not available.

The Rôle Played by pH Changes in the Apparent Carbonic Anhydrase Enzyme Activity of Zinc.

Since the test used depends essentially on a change in pH, any factor tending to alter the pH to any extent would cause changes in results indicative of activity, and it was thought possible that the pH changes caused by the zinc, although small, could adequately explain its apparent activity. In order to investigate this factor, experimental work was carried out and data were collected in order to construct three graphs.

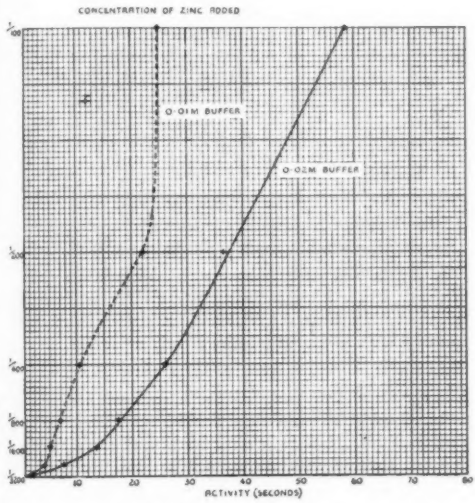

Fig. 1. The effect of zinc on the time taken for saturated CO_2 to reduce the pH of veronal buffer to pH 6·4. Details in text.

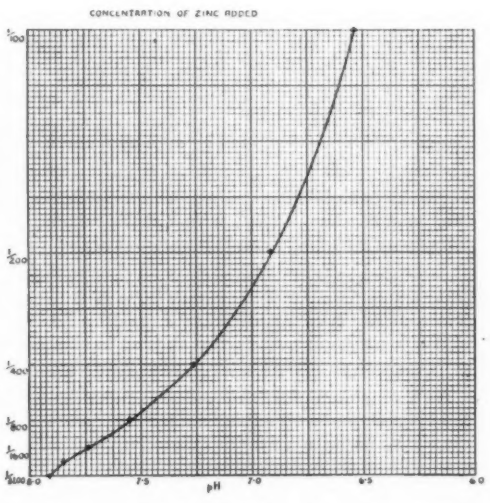

Fig. 2. The effect of zinc on the pH of veronal buffer. Concentration plotted against the pH change it causes. Details in text.

The first (Fig. 1) has the zinc concentration plotted against the "activity" in seconds. In the second (Fig. 2) the zinc concentration is plotted against the pH fall it caused, and in the third (Fig. 3), the effect of pH changes alone is plotted against the activity in seconds which these changes caused. On transferring results from one graph to the next, as will be shown later, it is possible to show how much effect the pH fall occasioned by the zinc accounts for the "activity" due to the zinc.

Fig. 1. Using 0·02 M veronal buffer (pH 7·88), the activity of zinc solutions added in concentrations of 1/100, 1/200, 1/400, 1/800, 1/1,600, 1/3,200 was determined in the manner described previously. The blank test took 70·9 seconds, whereas the ascending dilutions of zinc took 12·6, 34·2, 44·9, 52·0, 56·2 and 61·0 seconds respectively, indicating "activities" of 58·3, 36·7, 26·0, 17·5, 13·3 and 8·5 seconds respectively. The "activity" of the zinc is plotted against the concentration.

A second graph is constructed in the same way, this time using 0·01 M buffer. Here the blank was 31·5 and "activities" 25·2, 21·8, 10·7, 7·0, 5·1 and 4·1 seconds respectively. This is also shown in Fig. 1 and the necessary data for both graphs are tabulated in Table 2.

TABLE 2.

The effect of zinc in varying concentrations on the "activity."

Date	Blank	Zinc concentration added	Test (Av.)	Activity	
21/11/49	70·9	1/100	12·6	58·3	
21/11/49	70·9	1/200	34·2	36·7	
21/11/49	70·9	1/400	44·9	26·0	0·02 M Buffer
21/11/49	70·9	1/800	52·0	17·5	pH = 7·88
21/11/49	70·9	1/1,600	56·2	13·3	
21/11/49	70·9	1/3,200	61·0	8·5	
22/11/49	31·5	1/100	6·3	25·2	
22/11/49	31·5	1/200	9·7	21·8	
22/11/49	31·5	1/400	20·8	10·7	0·01 M Buffer
22/11/49	31·5	1/800	24·5	7·0	pH = 7·98
22/11/49	31·5	1/1,600	26·4	5·1	
22/11/49	31·5	1/3,200	27·4	4·1	

Fig. 2. In this case, 15 ml. of veronal buffer were mixed with 5 ml. of glass distilled water and then 5 ml. of zinc sulphate were added in the same concentrations used in the previous set of experiments, i.e. 1/100, 1/200, 1/400, 1/800, 1/1,600, 1/3,200. The pH of these various solutions was determined and the pH plotted against the zinc concentration. These results can be seen in Fig. 2 and the relevant data in Table 3.

TABLE 3.

The effect of zinc on the pH of veronal buffer.

Concentration zinc	pH
—	7·94
1/100	6·56
1/200	6·92
1/400	7·26
1/800	7·55
1/1,600	7·74
1/3,200	7·85

Fig. 3. Here the tests were set up as if an ordinary blank test for carbonic anhydrase activity were to be performed, but the solution (veronal buffer and glass distilled water) was placed in the electrode of the pH meter, this being packed with ice. CO_2 at 0° C. was added through the side arm, the pH fall noted and the time taken every 0·2 of a pH unit. Thus an idea of the fall in pH in terms of time and the general shape of the graph of the actual reaction was obtained. This was done, using 0·02 M buffer, the points plotted being the average points over a number of estimations of that particular fall in pH. Fig. 3 shows the pH plotted against the "activity." It was supposed that if the change from say pH 7·9 (pH of the buffer) to pH 7·5 took 25 seconds, then anything which caused a change in pH of this magnitude would be expected to have an "activity" of 25 seconds entirely due to its pH effect. Table 4 shows the relevant data.

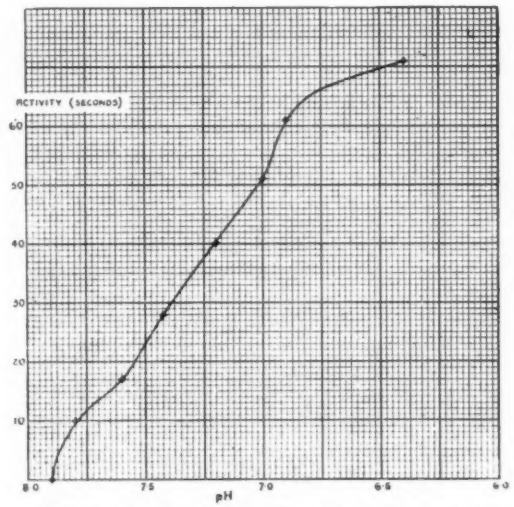

Fig. 3. The "activity" of blank tests due to pH changes. The fall in pH is plotted against the time taken for that fall to occur. Details in text.

It is significant to note in Fig. 3 that the most rapid changes in time occur early in the reaction with relatively small changes in pH. This, of course, is of great importance in any assessment of the validity of the test since it is readily recognizable that small changes in pH cause relatively large changes in time, and it is in this range that small changes in pH are very likely to occur. It is necessary to have the solution extremely well buffered and the veronal buffer used (0·02 M) is really not adequate to ensure a sufficiently constant pH. It seems, therefore, that when using this test with solutions likely to alter the pH even to a small extent, care must be taken in interpreting results.

TABLE 4.

Time (seconds) taken for the pH to fall to the shown value.

pH*	7·8	7·6	7·4	7·2	7·0	6·8	6·6	6·4	6·2	6·0	5·8	5·6	5·4	5·2
	9	14	23	33	45	54	60	65	68	74	80	87	98	117
	10	17	27	38	49	58	64	68	72	75	79	86	96	117
	11	18	30	41	51	59	66	71	75	79	84	92	105	—
Time (seconds)	11	16	25	38	49	60	68	72	76	81	89	98	111	—
	9	18	32	45	57	68	75	80	85	89	93	100	111	—
	9	16	26	40	51	61	66	71	75	80	86	95	108	
	12	22	35	48	57	66	71	75	79	84	92	103	122	
Average time (sec.)	10	17	28	40	51	61	67	71	76	80	85	92	107	117

* Commencing pH = 7·9.

On examination of the three graphs in turn and on transferring results from one to the other, it can be seen that the pH change caused by the zinc adequately explains any apparent "activity" possessed by this metal. Thus from Fig. 1 it is seen that a concentration of zinc of 1/800 possesses an "activity" of 18 seconds. However, from Fig. 2 this concentration is shown to cause a pH change from 7·9 to 7·55 and on examining this pH change in Fig. 3 it can be seen to have an "activity" of 20·5 seconds. Using a zinc concentration of 1/3,200 and working as before from the graphs, the "activity" is 9 seconds (Fig. 1), while the pH fall is from 7·9 to 7·85 (Fig. 2), accounting for an "activity" of 5 seconds (Fig. 3). With the higher concentrations of zinc the correspondence is again noted, 1/400 giving an "activity" of 26 seconds (Fig. 1) and a pH fall to 7·25 (Fig. 2), which accounts for an "activity" of 37 seconds. Thus it appears that over the whole range studied the pH change occasioned by the zinc is adequate to explain any apparent activity of this metal.

In confirmation of this finding it was decided to set up a series of tests containing zinc to determine the "activity" and the pH fall caused by that concentration and then set up another series in which the pH was altered over the same range by the addition of 0·1 N HCl and to determine the "activity" of these solutions and so confirm the fact that pH changes explain fully the effect of the zinc. For this purpose three solutions were prepared, as follows:

Solution I. 75 ml. veronal buffer (0·02 M) + 25 ml. 1/800 Zn.
Solution II. 75 ml. veronal buffer (0·02 M) + 25 ml. 1/1,600 Zn.
Solution III. 75 ml. veronal buffer (0·02 M) + 25 ml. 1/3,200 Zn.

The pH of these solutions was found to be

$$I = 7{\cdot}25,\ II = 7{\cdot}58,\ III = 7{\cdot}65.$$

These solutions were used as the test solutions and their carbonic anhydrase "activity" determined in the usual way and compared with the "activity" of a blank containing glass distilled water in the place of the zinc solution. The results are shown in Table 5 and it is seen that the "activities" are 27·8, 13·7, and 11·1 seconds respectively.

The three solutions in which N/10 HCl was used to reduce the pH were prepared as follows:

Solution IV. 75 ml. veronal buffer (0·02 M) + 20 ml. glass distilled water + 6 ml. 0·1 N HCl. pH = 7·15.
Solution V. 75 ml. veronal buffer + 22 ml. glass distilled water + 3 ml. 0·1 N HCl. pH 7·49.
Solution VI. 75 ml. veronal buffer + 25 ml. glass distilled water + 2 ml. 0·1 HCl. pH 7·49.

Again the "activities" of these solutions were determined and compared with a blank, exactly as was done for the zinc solutions. These values were 46·0, 19·2 and 19·6 seconds respectively. The data are shown in Table 5. It is seen that when the pH is reduced by HCl to a corresponding degree to the change caused by zinc, then HCl causes a similar change in the "activity," and if points are superimposed on Fig. 3 representing the "activity," against the pH change, then no matter whether the pH change be caused by HCl or zinc, the points fall approximately along the curve of Fig. 3.

TABLE 5.

		Blank (seconds)	Test (seconds)	Activity (seconds)	pH
Zinc solutions	I	78·3	50·5	27·8	7·25
	II	73·4	59·7	13·7	7·58
	III	73·4	62·3	11·1	7·65
HCl solutions	IV	78·3	32·3	46·0	7·15
	V	73·4	54·2	19·2	7·49
	VI	73·4	53·8	19·6	7·49

These observations raise a serious objection to the validity of this method of carbonic anhydrase assay and since it can be used for blood cell estimations, it seemed profitable to determine whether cells in the concentrations used altered the pH and so made interpretation of results difficult. For this purpose rat blood drawn from the heart immediately after death was used and a dilution of 100 p.p.m. of the red cells made. This possessed considerable enzymic activity, sufficient to reduce the time from 66·3 seconds to 33·4 seconds, an "activity" of 32·9 seconds. However, when the pH of the buffer was determined, it was found to be 7·75 and that of the 100 p.p.m. cells (3·3 ml. cells + 10 ml. veronal buffer) to be also 7·75. Thus the cells in this concentration fail to alter the pH and the test remains valid for these estimations. However, in the case of substances likely to cause changes in pH, even of a small magnitude, the test would be invalidated to a certain extent.

DISCUSSION.

It has been shown that zinc reduces the pH of the veronal buffer used for the assay of carbonic anhydrase. This change in pH is sufficient to explain fully the apparent carbonic anhydrase activity possessed by zinc, when tested by this method. It is obvious that these observations and their implications raise an objection to the validity of this test and its range of usefulness. It can be seen from Fig. 3 that small changes in pH will cause a difference of some seconds in the reaction time and so if substances are used which are likely to alter the pH even slightly, then erroneous conclusions could be deduced. Zinc and other metallic ions are just such substances and even in relatively small concentrations cause small changes in pH which at first glance would appear too small to affect the result. However, when the facts are examined further, they are found sufficient to account entirely for any changes observed. After all, the veronal buffer used is not of high concentration (0·02M) and is

not of sufficient strength adequately to buffer many substances. These same objections could be raised to any test employing pH changes as an index of activity and possibly apply equally well to the method of Philphot and Philphot (1936) for carbonic anhydrase assay. It is important therefore, to realize the significance of these small pH changes and adequately eliminate or allow for them in the interpretation of experimental data. Blood cells, however, in the concentration used for assaying in this test have been shown to cause no change in the pH and so the complications do not arise in the case of blood cells. It would be expected that cells which are well buffered at a pH over the range 7·2–7·4 would reduce the more alkaline pH of the relatively weak veronal buffer. However, the concentration of cells appears insufficient to do this.

SUMMARY.

The apparent carbonic anhydrase activity of solutions of zinc when tested by the pH change method of Roughton and Booth (1946) was investigated and found to be due entirely to the small pH changes caused by this metal.

The validity of the test was investigated and discussed, both in use with blood cells and for other substances in which pH changes of small magnitude were likely to occur and its value found to be greatly curtailed when using substances which caused even slight pH changes.

Acknowledgments. I wish to express my indebtedness to Mr. D. I. B. Kerr, of this Department, for his help and encouragement during the course of this work, and to Dr. F. Lippay, for the use of the pH meter, which was used extensively in this investigation.

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INTERFERENCE BETWEEN INACTVE AND ACTIVE INFLUENZA VIRUSES IN THE CHICK EMBRYO

IV. THE EARLY STAGES OF VIRUS MULTIPLICATION AND INTERFERENCE

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(Accepted for publication 6th June, 1950.)

Hoyle (1948) reported that following injection of influenza virus into the allantoic sac of developing chick embryos the concentration of virus in the allantoic fluid became reduced, presumably as a result of absorption of virus by the allantoic cells; however, the virus was detectable by infectivity and haemagglutination in only small amounts in ground suspensions of the chorio-allantoic membrane. A similar observation was made independently by Henle (1949). In each case it was noticed that virus became detectable in the membrane in large amounts following a lag phase of 4–5 hours. Analogous results have been described by Rake and Jones (1942) for lymphogranuloma virus, by Heinmets and Golub (1948) for psittacosis virus, by Schlesinger (1949) for encephalomyelitis virus growing in mouse brain, and by Fazekas de St.Groth and Donnelley (1950) for influenza virus in mouse lungs.

The apparent disappearance of influenza virus during the lag phase might be due to combination of virus with an inhibitor, or to transformation of virus into an alternative form lacking agglutinating and infective properties. In an accompanying paper (Edney and Isaacs, 1950) the presence of an inhibitor of influenza virus haemagglutination in suspensions of normal chorio-allantoic membranes is described. This inhibitor was present in greatly diminished amount in eggs infected with influenza virus, the reduction in inhibitor occurring along with the appearance of haemagglutinin in the membrane. In addition, the inhibitor was inactivated by the receptor-destroying enzyme of *V. cholerae* (RDE). It was of interest, therefore, to see whether the disappearance of demonstrable virus might be due to the formation of a virus-inhibitor complex in the membrane which could be dissociated by RDE. The present paper reports an investigation of the "disappearance" of influenza virus following injection into the allantoic sac. The results allow some limited conclusions to be reached on the mechanisms of interference and multiplication in influenza viruses.

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Materials and Methods.

Most materials and methods used are described in the preceding paper.

Virus. MEL (*m*). Variant of Melbourne strain of influenza A, previously described (Isaacs and Edney, 1950a). This variant was used because it was found to be very susceptible to interference by heated LEE.

Antiserum. Antiserum was prepared by bleeding a ferret 10 days after intranasal inoculation of 0·25 ml. of 1:10 dliution of LEE allantoic fluid.

Antihaemagglutinin Titration.

Serum was inactivated shortly before use by diluting 1 in 5 in normal saline and heating at 62° C. for 15 minutes. Serial dilutions (0·25 ml.) were prepared in normal saline and an equal volume of 1 p.c. fowl cells was added. A third volume containing 5 A.D. of the test virus was added, mixed, and the cells allowed to settle at room temperature. Readings were made by the pattern method and the amount of serum causing partial agglutination was taken as the end-point. Antihaemagglutinin titres are expressed as the reciprocal of the initial dilution of serum present at the end-point.

Determination of Virus Enzymic Activity.

The method was based on that described by Burnet (1949). To 1 ml. of membrane suspension virus was added, to a final concentration of 100 A.D. per ml., with penicillin as a bacteriostatic agent. This was incubated for 18 hours at 37° C. The suspension was then diluted 1 in 10 in normal saline and heated at 65° C. for 30 minutes in order to inactivate virus haemagglutinin. Finally, the suspension was titrated for its inhibitor content against 5 A.D. of treated MEL virus (T-MEL). A second portion incubated at 0° C. but treated similarly otherwise, served as a control, since enzymic activity is negligible at that temperature.

EXPERIMENTAL.

"Disappearance" of Haemagglutinin During the Lag Phase.

Experiments were carried out in an attempt to detect haemagglutinin in the chorio-allantoic membrane during the lag phase.

Eleven-day eggs were injected allantoically with a large inoculum (one ml. of undiluted allantoic fluid) of active MEL virus. After two hours' incubation at 35° C. to allow adsorption of seed virus, the cavity was drained and the haemagglutinin content of the fluid determined. The allantoic sac was then washed out thrice with 5 ml. saline and the allantoic fluid replaced by 5 ml. of RDE diluted 1 in 5 in saline (4,000 units) to remove virus superficially adsorbed to the surface of the allantoic cells. After one hour's incubation at 37° C. the membranes were removed, ground, suspended in 1 ml. saline and lightly centrifuged. 0·25 ml. RDE was added to the supernatant and incubated for one hour at 37° C. in order to inactivate inhibitor. The fluid was then titrated for haemagglutinin, dilutions being made in 2 p.c. citrate-saline in order to stop further RDE action. In no case was haemagglutinin recovered in the lag phase (less than 1 A.D.).

It was calculated that approximately 70–200 A.D. of virus were absorbed by the membranes in different experiments: hence the recovery of haemagglutinin was less than one p.c. of the virus absorbed by the membrane. Some of the virus is taken up by the visceral portion of the allantoic membrane, but although this has not been taken into account in the calculations, it would not affect the results greatly. Controls showed that a suspension of ground

normal chorio-allantoic membrane had a weak inhibitory action on haemagglutination by active MEL virus. However, following incubation of comparable amounts of MEL virus and membrane suspension for one hour at 37° C. *in vitro* with subsequent treatment with RDE, there was 80 p.c. recovery of haemagglutinin. Thus the combination of MEL virus and inhibitor is largely reversible by RDE *in vitro*.

Infectivity of Virus in the Chorio-Allantoic Membrane During the Lag Phase.

The results of Henle (1949) have shown that only 1–5 p.c. of injected virus can be detected in suspensions of chorio-allantoic membrane during the lag phase. Henle suggested that the small amount of recovered virus might have been superficially adsorbed to the surface of the allantoic cells. If this were so, incubating the chorio-allantoic membrane in RDE, and washing thoroughly, should reduce the recovery of virus following grinding.

Normal 11-day embryos were injected allantoically with 10^7 ID_{50} of MEL (*m*) virus (0·05 ml. of a 1:100 dilution of allantoic fluid). After 3 hours' incubation at 35° C. the allantoic fluids were harvested, their volume measured and their infectivity titrated in eggs. The chorio-allantoic membranes were removed and washed thoroughly in normal saline. Two treatments were then carried out. In the experimental group, the membrane was incubated for 30 minutes at 37° C. in 5 ml. of 1 in 5 RDE, then washed thoroughly in saline, and again incubated in the same amount of RDE and washed. This step was omitted in the control group. In each case the membrane was ground, suspended in 1 ml. saline, and lightly centrifuged. 0·5 ml. RDE (2,000 units) was added to the supernatant and incubated one hour at 37° C. (This is greatly in excess of the amount of RDE required to inactivate inhibitor from the membrane in this time.) The membrane suspension was then titrated for infectivity in eggs. Insufficient RDE was present to interfere with the results of the infectivity titrations. The results of representative experiments are shown in Table 1.

TABLE 1.

Recovery of virus from allantoic fluid and chorio-allantoic membrane 3 hours after allantoic injection.

Dilution of material	Egg infectivity titration			
	Experimental		Control	
	Allantoic fluid (7 ml.)	Membrane suspension	Allantoic fluid (6 ml.)	Membrane suspension
10–1	—	6/6	—	6/6
10–2	—	2/6	6/6	6/6
10–3	5/6	1/6	5/6	4/6
10–4	3/6	0/6	3/6	1/6
10–5	0/6	—	0/6	0/6
Per cent. seed virus recovered	12 p.c.	0·04 p.c.	15 p.c.	0·6 p.c.

Figures express results of egg infectivity titrations: numerator gives number of positive eggs and denominator, number inoculated.

Experimental: chorio-allantoic membrane incubated in RDE before grinding. Membrane suspensions were incubated in RDE after grinding in experiment and control.

As described by Hoyle (1948) and Henle (1949), the amount of virus recovered from ground suspensions of chorio-allantoic membrane was very much less than the amount absorbed by the membrane. The recovery of virus found in Hoyle's and Henle's experiments was not increased by treating the ground membrane suspension with sufficient RDE to inactivate all inhibitor present. Also, incubation of the membrane with RDE before grinding caused a significant reduction in the amount of virus recovered, suggesting, in agreement with Henle, that much of the virus recovered at first was superficially adsorbed to the membrane.

Ground suspensions of normal chorio-allantoic membrane incubated along with active virus *in vitro* do not decrease the infectivity of the virus. (Hoyle, 1948; Henle, 1949; Edney and Isaacs, 1950).

"Disappearance" of Inactive Virus Haemagglutinin Following Absorption by the Chorio-Allantoic Membrane.

Experiments were carried out to see whether haemagglutinin could be detected in suspensions of ground chorio-allantoic membrane following injection of inactive virus. Since no multiplication of virus occurred it was possible to incubate the eggs for periods of time longer than the lag phase for active virus (4–5 hours), and to use large inocula. Combination of heated LEE with the inhibitor in normal allantoic fluid was excluded by preliminary washing of the allantoic cavity and treatment of all samples with RDE.

The allantoic fluids of normal 11-day embryos were removed and the allantoic cavities washed thrice with 5 ml. saline, in order to remove normal allantoic fluid inhibitor of heated LEE. After the third wash, 5 ml. of heated LEE virus, 8,000 A.D. were injected and the eggs incubated at 35° C. for 24 hours. 1 ml. of allantoic fluid was then removed, incubated for 30 minutes at 37° C. with 1 in 10 RDE and titrated for haemagglutinin. In most experiments the virus was almost completely absorbed, as described previously (Isaacs and Edney, 1950c). The eggs were next injected with 1 ml. RDE and incubated for 30 minutes at 37° C., in order to remove virus superficially adsorbed to the surface of the allantoic cells, and the allantoic fluids were harvested. The membranes were then removed, ground, and treated with 0·5 ml. RDE. Haemagglutinin titrations were carried out on the allantoic fluid samples and the membrane suspensions, and the recovery of virus in each sample was calculated.

The results are shown in Table 2.

TABLE 2.

Attempted recovery of inactive virus haemagglutinin from chorio-allantoic membrane following allantoic injection.

Amount of virus lost from allantoic fluid	99·5–99·75 p.c.
Amount of virus recovered from allantoic cell surface	<0·1 p.c.
Amount of virus recovered from ground chorio-allantoic membrane	<0·1 p.c.

Inoculum: 5 ml. of heated LEE, 8,000 A.D.

It can be seen that after 24 hours' incubation more than 99 p.c. of the inoculated virus has disappeared, presumably as a result of absorption by the chorio-allantoic membrane, and that no virus could be demonstrated on the surface of the allantoic cells. A similar result was found when the chorio-allantoic membranes were removed from normal 11-day embryos, and added within a few minutes to heated LEE *in vitro*, i.e. the membranes absorbed virus which could not be recovered from them following grinding and treatment with RDE. Thus, although inactive virus is taken up by the allantoic cells in large amount, haemagglutinin could not be demonstrated in membranes which were ground and treated with RDE: inactive virus therefore behaved similarly to active virus in this respect.

In the accompanying paper it is shown that a suspension of normal chorio-allantoic membrane has a marked inhibitory action on haemagglutination by heated LEE, and that the inhibitory action is destroyed by incubating the membrane with RDE *in advance*. However, when a suspension of normal chorio-allantoic membrane was incubated with varying amounts of heated LEE at 37° C. *in vitro* for one hour, *followed* by incubation with RDE, the recovery of haemagglutinin was 10–25 p.c. of the amount originally present. The recovery was not reduced by prolonged incubation (24 hours) of virus and membrane at 37° C., evidence against any inactivation of virus *in vitro*.

This indicates that *in vitro*, the combination of membrane inhibitor and inactive virus is less readily dissociated by RDE than that between inhibitor and active virus. However, the recovery of haemagglutinin in eggs injected with comparable amounts of heated LEE *in vivo* was less than 0·1 p.c. (Table 2). It seems either that the combination of heated LEE and membrane inhibitor *in vivo* is less readily dissociated by RDE than *in vitro*, or that changes in the virus haemagglutinin occur *in vivo* which do not occur *in vitro*.

Attempted Demonstration of Virus Enzymic Activity in the Chorio-Allantoic Membrane During the Lag Phase.

Since haemagglutinin and infectivity could not be found in significant amounts in membranes which had absorbed influenza virus, attempts were made to detect virus by means of its other known characters. It has been shown that influenza viruses can inactivate enzymically the membrane inhibitor *in vivo* and *in vitro* (Edney and Isaacs, 1950). Experiments were carried out to see whether any evidence of virus enzymic activity could be found in the chorio-allantoic membrane during the lag phase following injection into eggs of active virus.

In these experiments virus was injected into the allantoic cavity and, after sufficient time was allowed for the membranes to absorb seed virus, the membranes were removed, ground, and incubated at 37° C. If any virus enzymic activity was present, the inhibitory content of the membrane would be reduced as compared with that of a control portion kept at 0° C. However, some method was required of removing virus adsorbed to the surface of the allantoic cells as

this would interfere with the experimental results. RDE could not be used and specific antiserum was therefore tried. This was not successful since control experiments showed that normal chorio-allantoic membranes absorbed sufficient antibody *in vitro* to inhibit the enzymic action of added virus. Surface virus was therefore removed from the membranes as far as possible by eluting into normal saline.

Six 11-day eggs were injected suballantoically with 0·5 ml. of active MEL virus (1,120 A.D.) and incubated at 35° C. for 2 hours. Samples of allantoic fluid were withdrawn at 2 minutes and 120 minutes and titrated for haemagglutinin; addition of RDE to these samples was not necessary as normal allantoic fluid does not significantly inhibit haemagglutination by this virus. With a knowledge of the volume of allantoic fluid the absorption of haemagglutinin was calculated to be between 400 and 700 A.D. per membrane.

The membranes were removed and incubated separately in 5 ml. normal saline for 30 minutes at 37° C. to allow elution of adsorbed virus; the incubation was repeated three times more in fresh changes of saline. The membranes were then ground, suspended in 1 ml. saline, lightly centrifuged and the supernatants removed. Half of each membrane was incubated for 18 hours at 37° C. and half at 0° C. as control, with penicillin added. Finally the membranes were titrated for inhibitor content against 5 A.D. of T-MEL virus. As a further control, active MEL virus was added to a normal membrane suspension *in vitro*, to a final concentration of 100 A.D./ml., roughly one-fifth of the calculated amount of virus absorbed by the membrane *in vivo*. The results are shown in Table 3.

TABLE 3.

Lack of enzymic activity of virus in the chorio-allantoic membrane during the lag phase.

Suspension incubated at	Inhibitory titre of membrane						
	1	2	3	4	5	6	Control
37° C. (experimental)	240	320	240	320	320	320	25
0° C. (control)	240	400	440	320	480	720	720

Membranes 1–6 — virus injected *in vivo*.
Control membrane — virus added to membrane *in vitro*.
Inhibitor was titrated against 5 A.D. of T-MEL.

It can be seen that no significant reduction in inhibitory titre of the six suspensions occurred, following incubation for 18 hours at 37° C., compared with the titre of the samples kept at 0° C. The slight drop in titre found in three cases can be accounted for by the action of small amounts of virus still adsorbed to the membrane surface before grinding. In control experiments with this seed, it was found that an average of 7 p.c. of the virus adsorbed by the membrane remained on the surface of the cells and could be removed by RDE; presumably the remaining 93 p.c. is taken into the cells. However, the results in Table 3 show that this absorbed virus (93 p.c. of 400 A.D., or roughly 370 A.D.) has no significant enzymic activity. In contrast, a control in which a smaller amount of virus (100 A.D.) was added *in vitro* showed a reduction in inhibitory titre of the membrane of more than 95 p.c. (Table 3). There is thus no evidence of enzymic activity of virus in the membrane during the lag phase.

Antibody-Combining Power of Membranes Injected with Influenza Virus.

It was mentioned earlier that after mixture of membrane suspension and heated LEE *in vitro*, followed by incubation with RDE, only 10–25 p.c. of the haemagglutinin present was demonstrable. However, when specific LEE antiserum diluted 1 in 32 was added to a mixture of heated LEE and membrane *in vitro*, followed by RDE to inactive membrane inhibitor, there was a drop in the antihaemagglutinin titre of the serum against 3 A.D. of LEE virus, indicating combination of the heated LEE and antibody. The drop in titre from 12 to 3 (see Table 4), reflected 100 p.c. of the amount of heated LEE originally present in the mixture, as shown by controls of heated LEE and antiserum alone. The reaction might be represented by the equation

(Heated LEE + membrane inhibitor) + antibody = (Heated Lee + antibody) + membrane inhibitor.

Addition of RDE would then inactivate the free membrane inhibitor. The combination of heated LEE and antibody is shown by the fact that the mixture gives a reduced antihaemagglutinin titre against LEE virus, compared with controls without heated LEE. Since it appeared from these results that the combination of heated LEE and membrane inhibitor was only partially reversible by RDE but wholly reversible by antibody, it was of interest to test the antibody-combining power of membranes from eggs which had been injected with heated LEE *in vivo*.

Eleven-day eggs were washed with saline and injected with 5 ml. of heated LEE (9,600 A.D.), as described above. After incubation overnight the membranes were removed, ground, and saline suspensions prepared from them. LEE antiserum 1/32 was added and incubated for 30 minutes at 37° C. Finally 0·5 ml. RDE was added and incubated for 30 minutes at 37° C. in order to inactivate residual inhibitor.

Antiserum diluted in saline was used as control. The antihaemagglutinin titres were tested against 3 A.D. of LEE. Table 4 shows the combined results of this experiment and of the above experiment in which the membrane and heated LEE were mixed *in vitro*.

TABLE 4.

Antibody-combining power of heated LEE and chorio-allantoic membrane suspension.

Assay	Antihaemagglutinin titre
Antiserum + (heated LEE + membrane *in vitro*)	3
Antiserum + heated LEE control	3
Antiserum + (heated LEE + membrane *in vivo*)	12
Antiserum + saline control	12

In vivo and *in vitro* describe the experimental conditions under which the heated LEE and membrane were mixed (see text).

The antihaemagglutinin titres were tested against 3 A.D. of LEE virus.

It can be seen from Table 4 that a drop in antihaemagglutinin titre occurred in the *in vitro* experiment but not in the *in vivo* experiment. The

same results were obtained in experiments with active MEL virus. It appears therefore that membranes which have absorbed influenza virus *in vivo* show no evidence of antibody-combining power.

Antigenicity in Mice of Membranes which have Absorbed Inactive Virus.

A second method of testing for the presence of virus antigens in membranes which had absorbed influenza virus was to determine their antigenicity in mice, whose sera were then examined for the development of antihaemagglutinin.

The technique used was to inject washed eggs with 5 ml. of inactive virus (4,000 A.D. of heated LEE) allantoically, and incubate for 18–24 hours at 35° C. to allow the membranes to absorb more than 99 p.c. of the inoculum. The membranes were removed and soaked in RDE at 37° C. in order to release virus superficially adsorbed to the membrane. They were then washed, ground and suspended in 1 ml. saline per membrane and lightly centrifuged. 0·25 ml. of the supernatant was injected intraperitoneally into 9-weeks-old mice. Controls consisted of mice injected with 0·25 ml. of a suspension of normal chorio-allantoic membrane, heated LEE alone, and heated LEE mixed with normal membrane suspension *in vitro*.

After 14 days the mice were bled. The sera were diluted 1 in 5 with normal saline and heated at 62° C. for 15 minutes in order to inactivate non-specific inhibitor of haemagglutination. The sera were then tested for their power to inhibit agglutination by 5 A.D. of LEE virus. The results are shown in Table 5.

TABLE 5.

Immunization of mice with membranes which have absorbed heated LEE.

Mouse serum	Antihaemagglutinin titre			
	A	B	C	D
1	<10	<10	10	60
2	<10	<10	40	30
3	<10	<10	17	60
4	<10	<10	60	60
5	<10	—	40	60
6	<10	—	15	40
7	<10	—	30	30
8	<10	—	30	35
G.M. ± S.E.	<10	<10	26 ± 6	43 ± 5

Experimental group A: mice injected with membranes which had adsorbed heated LEE *in vivo*.
Control group B: mice injected with normal membrane.
Control group C: mice injected with heated LEE.
Control group D: mice injected with heated LEE mixed with normal membrane *in vitro*.

The results show that the experimental mice (group A) did not show any antihaemagglutinin response, although if all the virus inoculated had been taken up by the membranes, they would have received five times more virus than the mice in the control groups C and D. A similar experiment in which

6,000 A.D. of heated MEL virus were used in place of heated LEE, gave the same result. There is therefore no evidence of virus haemagglutinating antigen in chorio-allantoic membranes following absorption of inactive virus.

Interference Experiments with Membranes which have Absorbed Inactive Virus.

It has been shown previously that heated LEE injected into eggs allantoically interferes with the multiplication of active virus. The *minimal interfering dose* of heated LEE was found to be approximately 500–1,000 A.D. (Isaacs and Edney, 1950b). When this amount of heated LEE was mixed with a suspension of normal chorio-allantoic membrane *in vitro*, the interfering action of the heated LEE persisted, although it was not so marked as with heated LEE alone. Normal membrane suspension alone had no interfering action. Accordingly membranes which had each absorbed 9,600 A.D. of heated LEE *in vivo* (as described above) were tested for their interfering power. One quarter of a membrane, containing theoretically 2,400 A.D. of heated LEE, was used as the "interfering" inoculum in each egg, but no interference was demonstrated with the multiplication of 100 ID_{50} of MEL virus.

"Disappearance" of Active Virus in Eggs Previously Injected with Interfering Virus.

Since active virus could not be detected in suspensions of chorio-allantoic membrane during the lag phase and since inactive virus is known to interfere with the multiplication of active virus, experiments were carried out to see whether inactive virus prevented the "disappearance" of active virus in the membrane.

Eleven-day embryos were injected suballantoically with a *minimal interfering dose* of heated LEE (600 A.D.) and incubated at 37° C. for one hour. They were then injected with 10^7 ID_{50} of MEL (*m*) virus and the infectivity of virus in the chorio-allantoic membrane during the lag phase determined as described above. The results of a representative experiment are shown in Table 6.

TABLE 6.

Effect of interfering virus on recovery of active virus from allantoic fluid and chorio-allantoic membrane 3 hours after suballantoic injection.

Recovery of virus from	Percentage of seed virus
Allantoic fluid	13 p.c.
Chorio-allantoic membrane suspension	0·06 p.c.

Interfering virus: 600 A.D. of heated LEE.
Seed virus: 10^7 ID_{50} of MEL (*m*).

The results in Table 6 are similar to those in Table 1. As a control it was shown that 600 A.D. of heated LEE interfered with the multiplication of 10^7 ID_{50} of MEL (*m*) virus, producing a reduction in the haemagglutinin titre of a group of eggs from a mean of 433 to one of 18. Thus an amount of heated LEE which caused marked inhibition of the multiplication of 10^7 ID_{50} of MEL virus did not prevent the "disappearance" of active virus.

DISCUSSION.

This study was stimulated by Hoyle's (1948) and Henle's (1949) observations that influenza virus injected into the allantoic cavity could be detected in suspensions of ground chorio-allantoic membrane during the lag phase in very small amount only. The present results show that active and inactive (heated) virus behaved alike in this respect and that the use of RDE did not increase the yield of virus. Following absorption of active or inactive virus by chorio-allantoic membranes, membrane suspensions were tested for six known properties of influenza viruses. There was no evidence that these suspensions showed any significant degree of infectivity for eggs, haemagglutinin, enzymic activity on the membrane inhibitor, antibody-binding power, antigenicity in mice as regards production of specific antihaemagglutinin, or ability to interfere with the propagation of active virus. The behaviour of comparable amounts of virus incubated with normal membrane suspensions *in vitro* was quite different. Here there was inhibition of haemagglutination particularly by inactive virus, but none of the other activities mentioned was markedly inhibited by the membrane suspension *in vitro*. It is difficult to account for the results found with intact membranes *in vivo* in terms of an inhibitor alone.

Any further idea of what occurs in the membrane after absorption of inactive or active virus must depend, at present, on speculation only. However, in view of the complete lack of evidence of any virus activity in the membrane during the lag phase following injection of active virus, the theory of Hoyle (1948) that virus might be broken down into smaller units on entering the cell is interesting; these, by analogy with bacteriophages, might be supposed to multiply and then recombine to form fresh virus particles. An additional hypothesis suggests itself, as a corollary to that of Hoyle, i.e. that inactive virus particles are taken up by the allantoic cells and broken down into smaller units which can compete with similar units of active virus for cell constituents required in virus synthesis.

Three stages in the initiation of infection by influenza viruses can now be recognized.

(1) Adsorption of virus to the allantoic cell surface.

(2) Entry of virus into the allantoic cells, presumably as a result of the process suggested by Fazekas de St.Groth (1948) i.e. *viropexis*.

(3) "Disappearance" of virus—possibly by breakdown into smaller units.

We have previously shown that following injection into eggs of a *minimal interfering dose* of inactive virus there was no inhibition of the uptake of active virus by the allantoic cells. It has now been found that in interference the active virus cannot be detected in the cells during the lag phase. It therefore appears that the interference process is at a later period in virus multiplication than the third stage.

SUMMARY.

Influenza virus injected into the allantoic sac was in large part absorbed by the chorio-allantoic membrane, but less than one p.c. of the inoculum, as measured by infectivity and haemagglutinin tests, was recovered from ground suspensions of the chorio-allantoic membranes following treatment with RDE.

The recovery of virus was reduced a further ten-fold, as measured by infectivity tests, if the membranes were incubated in RDE before grinding, in order to remove superficially adsorbed virus.

Inactive (heated) influenza virus behaved similarly to active virus; the recovery of virus haemagglutinin from chorio-allantoic membrane suspensions was less than 0·1 p.c. of the virus absorbed.

Following absorption of active or inactive virus by the chorio-allantoic membrane there was no evidence that membrane suspensions showed any viral enzymic activity, antibody-combining power, antigenicity in mice as regards production of specific antihaemagglutinin, or ability to interfere with the propagation of active virus.

The interference process did not inhibit the "disappearance" of active virus in the chorio-allantoic membrane.

Acknowledgment. We are greatly indebted to Professor F. M. Burnet for his advice and criticism.

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THE DETERMINATION OF INORGANIC PHOSPHATE IN THE PRESENCE OF LABILE ORGANO PHOSPHORUS COMPOUNDS[1]

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(Accepted for publication 29th June, 1950.)

For the study of biological systems involving phosphate transfer it is desirable to have an accurate method for the estimation of inorganic and labile phosphates. The method of Fiske and Subbarow (1925) has been modified by many authors but such modifications have been primarily concerned with a search for conditions under which preferential reduction of phosphomolybdic acid would take place in the presence of molybdic acid. None of these modifications is readily applicable in the presence of acid-molybdate labile compounds such as phosphocreatine, acetyl phosphate, or ribose phosphate although attempts have been made to overcome these difficulties by various procedures. An important modification of the original Fiske and Subbarow method was introduced by Lowry and Lopez (1946) who used ascorbic acid as a reducing agent at pH 3·5–4·2 in the presence of a low molybdate concentration. Under these conditions labile compounds are much more stable than under the conditions used by Fiske and Subbarow (1925). An objection to the method of Lowry and Lopez may be raised, however, on the basis of interfering substances since these authors state "in certain tissue extracts the reaction is delayed and an internal standard must be used".

Many of the disadvantages inherent in the Fiske and Subbarow method and modifications thereof have been discussed by Berenblum and Chain (1938 a) and these authors (1938 b) introduced a new principle in that the phosphomolybdic acid is removed from the aqueous phase by extraction with isobutanol. Interfering substances are thus largely left behind and reduction is carried out in the alcohol phase and in the absence of molybdic acid.

It occurred to us that, if the reaction between phosphate and molybdic acid was fast enough and that if the resultant phosphomolybdic acid could be extracted from the aqueous phase in a few seconds the contribution which a molybdate-labile organo phosphate could make to the true inorganic phosphate would be minimized. It is the purpose of this paper to detail the investigations

[1] The subject matter of this paper was presented at a meeting of the Australian and New Zealand Association for the Advancement of Science in Hobart in January, 1949.

which have been carried out on the reactions involved and to describe a rapid method for the determination of inorganic phosphate in the presence of certain acid-molybdate labile phosphorus compounds.

METHODS.

Reagents. (1) Sodium phosphocreatine—prepared as described by Ennor and Stocken (1947)—all solutions were made up in distilled water and the pH adjusted to about pH 7·5 by the addition of NaOH.

Measurement of colour intensities. All measurements were made in 1 cm. cells on the Spekker Photoabsorptiometer using Ilford Filter No. 604, Spectrum Red.

RESULTS.

In the original method as described by Berenblum and Chain (1938b) it was recommended that the concentration of H_2SO_4 and ammonium molybdate be 0·5 *N* and 1·25 p.c. respectively and that the phosphomolybdic acid be extracted by shaking the mixture of these reagents plus the aliquot containing the P with 10 ml. of iso-butanol for 1–2 minutes. The aqueous layer was then allowed to separate and was discarded. The alcohol layer was shaken for 30 seconds with two successive amounts of 10 ml. of *N* H_2SO_4 and then reduced by shaking for a further 30 seconds with $SnCl_2$. The alcohol layer was then made up to a definite volume with ethanol and the colour measured. With this method the contribution which a labile compound will make to the inorganic P present will depend on the length of time during which the aqueous phase is in contact with the butanol, the concentration of acid and of molybdate and on the temperature. The influence of each of these factors has been determined under a variety of circumstances.

Initially experiments were carried out to determine the minimum time necessary for the extraction of the phosphomolybdic acid with iso-butanol. These were done at 0° C. and at room temperature—about 20° C.—and in two series which were made up as set out in Table 1.

TABLE 1.

Contents of separating flasks for determination of time necessary for complete extraction of phosphomolybdic acid.

Reagent	Dilute reagents	Concentrated reagents
Ammon. Molybdate	1·0 ml of 1 p.c. (w/v)	2·5 ml. of 5 p.c. (w/v)
H_2SO_4	1·0 ml. of *N*	0·5 ml. of 10 *N*
NaCl*	2·0 ml. of 5 p.c. (w/v)	2·0 ml. of 5 p.c. (w/v)
H_2O and/or P to a final vol. of 10 ml.	4·0 ml.	4·0 ml.

* NaCl was added to increase the speed of separation of the aqueous and alcohol phases.

After the addition of the aliquot containing inorganic phosphate, the flasks were allowed to stand for 15 minutes to ensure completion of the reaction between P and molybdic acid. 20 ml. of iso-butanol were then injected into each flask, under pressure, from a syringe. The flasks were then vigorously shaken for varying time intervals and the aqueous layer, after separation, was immediately run off. The alcohol layer was then shaken twice (10 seconds) with two successive volumes each of 10 ml. of *N* H_2SO_4, and then with $SnCl_2$ of concentration similar to

that used by Berenblum and Chain (1938b). The alcohol layer containing the reduced phosphomolybdic acid was then made up to 25·0 ml. with ethanol washings of the separating funnel and the colour intensity measured.

TABLE 2.

Time necessary for the extraction of phosphomolybdic acid from the aqueous phase.

Dilute reagents		Concentrated reagents	
Time extraction (seconds)	P.c. extracted	Time extraction (seconds)	P.c. extracted
	Temperature 0° C.		
30	90	10	96
40	94	20	100
60	98		
	Temperature 20° C.		
5	90	3	95
10	100	5	100
		10	100

The results obtained show that at 20° C. the butanol is capable of extracting all the phosphomolybdic acid present in both series in 10 seconds. When the temperature is reduced to 0° C. extraction takes considerably longer to reach completion.

TABLE 3.

Rate of combination of phosphate and molybdate

Dilute reagents (60 seconds extraction time)		Concentrated reagents (20 seconds extraction time)	
Time of reaction (seconds)	P.c. combined	Time of reaction (seconds)	P.c. combined
	Temperature 0° C.		
60	82	30	81
90	93	60	91
120	97	90	93
		120	94
	Temperature 20° C.		
(10 seconds extraction time)		(10 seconds extraction time)	
15	90	10	82
20	100	15	100

The rate at which phosphate reacts with molybdic acid was then investigated at the same temperatures and using the extraction times found to be necessary for 100 p.c. extraction for each series at the appropriate temperatures. For this purpose the set-up of the flasks was altered from that shown in Table 1 although the actual volumes and concentrations of the reagents used remained constant. The aliquot containing phosphate was added but that containing the molybdate was omitted until the experiment was carried out. At 20° C. and in the

case of the 10 seconds time interval, the molybdate and the butanol were added simultaneously, under pressure from two syringes, and the flasks then rapidly stoppered and shaken vigorously for the remainder of the interval. Here, addition of the butanol and molybdate and stoppering was accomplished in 2 seconds. For periods longer than 10 seconds the molybdate was added at zero time and 15 seconds before the end of the period the butanol was injected—shaking was then carried out until the end of the period. In the case of the experiments at 0° C., the butanol was added at such a time as to allow of a 60 second extraction time. The subsequent treatment was similar to that described earlier.

The results (Table 3) indicate that the reaction is completed in about the same time in the presence of the dilute reagents as with the concentrated reagents at 20° C. At 0° C. the rates are again about the same although here completion of reaction was not achieved up to 2 min. which was the longest time interval investigated.

From the results already described it is clear that, at room temperature, and if *maximal values* for phosphomolybdic acid are required and dilute reagents used an overall reaction time of 20 seconds with an extraction time of 10 seconds is necessary. Since, however, the reaction has gone to over 80 p.c. completion in 10 seconds, it is also clear that the time of 20 seconds need not be adhered to. The standard conditions which yield 100 p.c. extraction (5 seconds) and reaction (20 seconds) at room temperature may be applied under most laboratory temperatures and must by definition give reproducible results. This of course follows from the fact that there is complete reaction and extraction.

The measurement of inorganic P by this method will, in the presence of an acid-molybdate-labile compound, give an inorganic P value greater than the true content by a figure equal to the per cent. hydrolysis of the labile compound under the conditions of the determination. As a measure of this error, the hydrolysis of phosphocreatine at various temperatures and at the two reagent concentrations has been determined.

In the experiments with the concentrated reagents the separating funnels contained 2·0 ml. of 5 p.c. (w/v) NaCl, 4·0 ml. of H_2O, and 1·0 ml. of 1×10^{-3} M solution of phosphocreatine. 0·5 ml. of 10 *N* H_2SO_4 was added from a syringe and this was followed *immediately* by 2·5 ml. of 5 p.c. (w/v) ammonium molybdate. Butanol was added from a syringe 30 seconds before the end of each particular interval and the flasks shaken for the remainder of the time. In the case of the 30 second time interval the addition of the molybdate and butanol was made simultaneously.

The influence of temperature on the hydrolysis of phosphocreatine is marked and the curves (Fig. 1) indicate that the half life of the compound in the strong reagents is about 1 minute, 2 minutes, 3 minutes and 25 minutes at 30°, 22°, 18° and 0° C. respectively. Furthermore, it is clear that the application of the method of Berenblum and Chain (1938b) with extraction times of 1–2 minutes will result in 40–66 p.c., 25–50 p.c., 10–30 p.c, and 0–1 p.c. hydrolysis of phosphocreatine at temperatures of 30°, 22°, 18°, and 0° C. respectively.

Some experiments have been carried out with similar extraction times and using the same amounts of phosphocreatine but in the dilute reagents.

As would be expected, on the basis of the accepted catalytic effect of molybdic acid on the breakdown of phosphocreatine, reduction of both the acid and molybdate concentration

has a pronounced effect. In Fig. 2 the curve obtained with the strong reagents at 22° C. is reproduced for the sake of comparison which indicates that, at this temperature, the reduction of the acid and molybdate concentration has effected an increase in the half life of from 2 to 6 minutes.

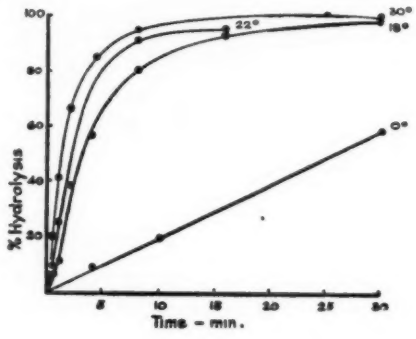

Fig. 1.

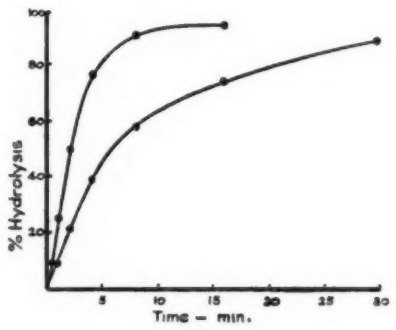

Fig. 2.

Fig. 1. Hydrolysis curves of phosphocreatine as measured at various temperatures and in the presence of concentrated reagents.

Fig. 2. Hydrolysis curves of phosphocreatine. The upper and lower curves represent the hydrolysis at 22° C. as measured in the presence of concentrated reagents and dilute reagents respectively.

Irrespective of whether dilute or concentrated reagents are used it is evident that the original method of Berenblum and Chain is subject to considerable error if phosphocreatine or any other molybdate labile compound is present. However, on the basis of the experiments designed to measure the reaction time of phosphate and molybdate and the extraction time with butanol it is possible to choose experimental conditions which will give maximal (or reproducible) results but which will not be subject to such large errors.

TABLE 4.

Contents of separating funnels for use in the determination of inorganic P.

Dilute reagents		Concentrated reagents	
NaCl (5 p.c. w/v)	2·0 ml.	NaCl (5 p.c. w/v)	2·0 ml.
H_2SO_4 (*N*)	1·0 ml.	H_2SO_4 (10 *N*)	0·5 ml.
KH_2PO_4 (10 μg./ml.)	0·5–2·5 ml.	KH_2PO_4 (10 μg./ml.)	0·5–2·5 ml.
H_2O	to 9·0 ml.	H_2O	to 7·5 ml.
Ammon. Molybdate (0·5 p.c. w/v)	1·0 ml.	Ammon. Molybdate (5 p.c. w/v)	2·5 ml.
iso-Butanol	20·0 ml.	iso-Butanol	20·0 ml.
Time of shaking	15 sec.	Time of shaking	10 sec.

Ammonium molybdate and butanol injected at zero time. Aqueous layer run off to waste immediately after end of extraction. Two washes, each of 10 seconds duration, with 10 ml. *N* H_2SO_4. Butanol layer after reduction to 25·0 ml. with ethanol.

TABLE 5.

Results of determinations of optical densities of varying amounts of standard P. Details as in Table 4.

Temperature 22° C.

	Dilute reagents (15 seconds extraction time)	Concentrated reagents (10 seconds extraction time)
μg. P	Optical density	Optical density
5	0·085, 0·085	0·095, 0·095
10	0·168, 0·168	0·182, 0·197
20	0·324, 0·340	0·374, 0·374
25	0·417, 0·409	0·467, 0·478

20 μg. P with strong reagents and 30 seconds extraction time gave an optical density of 0·405, 0·405 and in the weak reagents of 0·402 and 0·412.

The conditions chosen for investigation are set out in Table 4 and the results in Table 5. Under these conditions it is clear that excellent reproducibility is obtainable within each series and that the plot of the optical density v. μg. of P is linear. It should also be noted that the maximum result has not been achieved with these shortened times but as a linear response has been obtained this is of no importance.

In view of the results obtained with standard inorganic P the same conditions for reagent concentration and extraction times were chosen to determine the degree of hydrolysis of phosphocreatine.

TABLE 6.

Rate of hydrolysis of phosphocreatine in strong and weak reagents. Details as in Table 4.

Temperature 22° C.

Strong reagents			Weak reagents		
Reaction time (seconds)	Optical density	P.c. of maximum	Reaction time (seconds)	Optical density	P.c. of maximum
10	0·006, 0·008	1·4	15	0·007, 0·007	0·7
20	0·031, 0·035	6·4	30	0·028, 0·026	4·6
30	0·055, 0·065	12·0	45	0·042, 0·040	7·0

Note. Strong Reagents—At 10 seconds reaction time the butanol and molybdate were injected simultaneously. At the 20 and 30 seconds times the molybdate was injected at zero and the butanol at zero + 10 and zero + 20 seconds respectively, thus giving a standard 10 seconds extraction time. *Weak reagents*—A similar procedure was adopted here to give a standard extraction time of 15 seconds. *Phosphocreatine*—1·0 ml. of 1×10^{-3} M solution = 31 μg. of P.

The results (Table 6) show that the amount of P which is liberated in the shortest time interval investigated and in the strong reagents amounts to 1·4 p.c. of the total amount present.

The expression of the optical density as measured at any one time interval as a per cent. of the maximal obtainable under the conditions of the experiment is not necessarily a measure of

the percentage of hydrolysis. That this is true may be seen from the figures which show a departure from linearity due to several factors. Among these may be mentioned the reactions phosphocreatine → phosphate + creatine, phosphate + molybdate → phosphomolybdate and the butanol extraction rate. The amount of phosphate measurable at any one time interval will depend on the slowest of these reactions and thus a departure from linearity is to be expected particularly at the shorter time intervals. However, since the figures obtained for varying amounts of standard P are linear (Table 5) it follows that the methods adopted will give an accurate assessment of the magnitude of the error which is introduced in a measurement of inorganic P in the presence of phosphocreatine. Thus if inorganic P is being measured in such a system one would expect the value obtained to be high by a figure which will be approximately 1·4 p.c. of *the total amount of P which is present as phosphocreatine.*

The validity of this argument has been tested by carrying out estimations of inorganic P on a standard solution of KH_2PO_4 in the presence and absence of added phosphocreatine. The separating funnels were charged with reagents as indicated in Table 4, the only departure from this procedure being that in the case of those funnels containing phosphocreatine in addition to inorganic P, 1·0 ml. of 1×10^{-3} M solution of phosphocreatine was added and the volume of water reduced by an equivalent amount. As usual the phosphocreatine was added immediately prior to the estimation.

TABLE 7.

Recovery of inorganic phosphate in the presence of phosphocreatine.

Molar ratio $\frac{\text{Phosphocreatine}}{KH_2PO_4}$	P.c. recovery
0·31	102
0·62	98
0·93	104
1·55	103
3·10	106

The results (Table 7) show that the recovery of added inorganic phosphate conforms to expectations in view of the results given in Table 6. It is also of interest to note that phosphomolybdic acid has no apparent accelerating effect upon the decomposition of phosphocreatine (Table 8).

TABLE 8.

Estimation of labile phosphorus of phosphocreatine in the presence of inorganic phosphate.

Phosphocreatine added μ moles	Phosphocreatine found μ moles	P.c. recovery
(1) 0·1	0·1	100
(2) 0·1	0·1	100
(3) 0·3	0·28	96

Note. Experiments 1 and 3 conducted in the presence of 0·323 μ moles of inorganic P. Experiment 2 was conducted in the presence of 0·646 μ moles of inorganic P.

The only other method for the determination of inorganic P with which we are familiar and for which accurate results are claimed in the presence of labile phosphates is that of Lowry and Lopez (1946). Some experiments have been carried out using this method, and whilst excellent recoveries of inorganic P have been obtained in model systems, extremely erratic results were obtained in trichloracetic acid extracts of certain tissues. Even dilution of such extracts to the maximum degree compatible with the sensitivity of the method failed to yield satisfactory results in the case of liver extracts. The reason for this has been found to be due to the glutathione content of the extracts and it has been previously noted by Potter, LePage and Klug (1948) that glutathione is an inhibitor of the method advocated by Lowry and Lopez.

DISCUSSION.

Because of the rapidity with which phosphate reacts with molybdic acid and the fact that the partition coefficient of phosphomolybdic acid in a water-iso-butanol mixture is overwhelmingly in favour of the alcohol phase it is possible to extract in 10 seconds over 90 p.c. of the phosphomolybdic acid from an aqueous phase with the solvent. It has been shown that the method is sufficiently rapid to permit the determination of inorganic phosphate in the presence of phosphocreatine with an error equal to about 1 p.c. of the total amount of P present as phosphocreatine. Some experiments were carried out using acetyl phosphate but owing to the extreme lability of this compound in the presence of molybdic acid the method proved unsatisfactory. Since, however, this compound is generally believed to be absent from tissues or tissue extracts the value of the method is not reduced by this result.

As Berenblum and Chain (1938b) have pointed out the method is free from interference by a wide variety of compounds and in our hands no interfering substances have been found. In investigations concerned with phosphate distribution, it is the general practice to treat the extracts with Ba and so to divide the phosphates into Ba-soluble and Ba-insoluble fractions. Removal of Ba, by addition of Na_2SO_4, prior to analysis is always necessary if the usual methods of phosphate analysis are used and losses are inevitably caused by adsorption on $BaSO_4$. In the case of the Berenblum and Chain (1938b) method in its modification described here, removal of Ba is unnecessary. The $BaSO_4$ which is formed on addition of the sample to the reagents is largely removed in the two acid washes and in the aqueous phase after reduction. Final traces may be removed by filtration of the reduced butanol (after adjustment to 25 ml.) through a Whatman No. 1 filter paper.

It has been found convenient to mount the separating funnels in groups of 4 so that all shakings subsequent to the initial one may be carried out *en masse* in a suitably constructed shaking machine.

The use of the same funnel for extraction of the phosphomolybdic acid and reduction of the butanol layer by $SnCl_2$ has led to false colours due presumably to adsorption of $SnCl_2$ on the glass. Reproducible results are not possible unless the funnels are treated with concentrated HNO_3 between each estima-

tion, but this has been avoided by quantitative transferral of the butanol layer to a second set of funnels, reserved for reduction. This procedure eliminates false colours and reduces washing to a mere rinse with tap and distilled water between determinations.

SUMMARY.

A modification of the Berenblum and Chain (1938b) method in the estimation of inorganic P is described.

This modification permits the direct determination of inorganic phosphate in the presence of phosphocreatine.

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A NEW RESPIRATORY PUMP

by H. ADAMS[1], B. N. ELLIS[2] AND GEOFFREY KAYE[3]

(Accepted for publication 10th May, 1950.)

The writers contemplated a study of the design of respiratory valves. For this purpose, they needed a pump which would "breathe" through the valves under trial and enable their efficiency to be studied by oscillographic methods (Kaye, 1949). The respiratory pumps usual in laboratories do not, however, imitate human breathing closely. Their rate and stroke-volume are controllable, but they fail to reproduce the normal variation in respiratory velocity. This latter was studied exhaustively by Fleisch (1925) and was brought to the notice of anaesthetists by Macintosh and Mushin (1946). Briefly, the velocity of both inhalation and exhalation rises to a peak in the first half-second and then diminishes to zero over the ensuing second. The time-velocity curve of a complete respiratory cycle has therefore the form shown in Fig. 1. Fleisch points out that this form represents merely a fair average sample, the peak in many individuals being less pronounced.

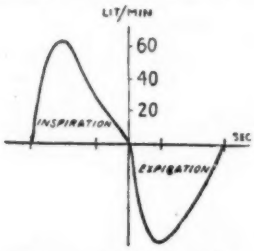

Fig. 1. Fleisch's Time-Velocity Curve of Respiration. Redrawn, from the original pneumotachograms, by Macintosh and Mushin.

For their proposed study, the writers needed a pump which would "breathe" like the human subject in a state of anaesthesia. The requirements appeared to be: (a) delivery in accordance with the Fleisch time-velocity curve, (b) controllable stroke-volume, representing "tidal respiration" variable between zero and one litre, (c) controllable rate, between 10 and 60 cycles per minute, (d) variable space above the piston at top dead-centre, representing the "dead-space volume" of anaesthetic apparatus. A pump which conforms to these requirements has been made and tested: it is described in this paper.

Construction of the Prototype.[4]

The pump depicted in Fig. 2 is merely a prototype, the product of trial-and-error. Since it delivers in conformity with the Fleisch time-velocity curve, as will be shown in this paper, it can be used in the writers' further studies. It was fabricated, however, in a private workshop

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of limited equipment and therefore suffers from mechanical defects, such as belt-slip and inadequate rigidity. It will hence be described only briefly, to show its essential mechanical principles. A fully-equipped workshop could, with but little extra trouble, produce a more compact and rigid version. A tentative design for one is included in this paper.

The general principles will be apparent from Fig. 2. The motive power was a single-phase electrical motor, developing ¼ horsepower at 1,440 revolutions per minute. Reduction of the speed by gearing was therefore imperative. By appropriate choice of pulleys, the final speed of the cam-shaft could be 10, 20, 30, 40 or 60 revolutions per minute, which seemed adequate for the purpose in view. The belt between the secondary countershaft and the cam-shaft was left overlong, tension being maintained by means of a jockey-pulley run on an eccentric lever-mounting. This arrangement was necessary to enable the belt to be changed from one step of the cone-pulleys to another, since the enmeshed worm and wheel included in the drive precluded backwards rotation of the secondary countershaft.

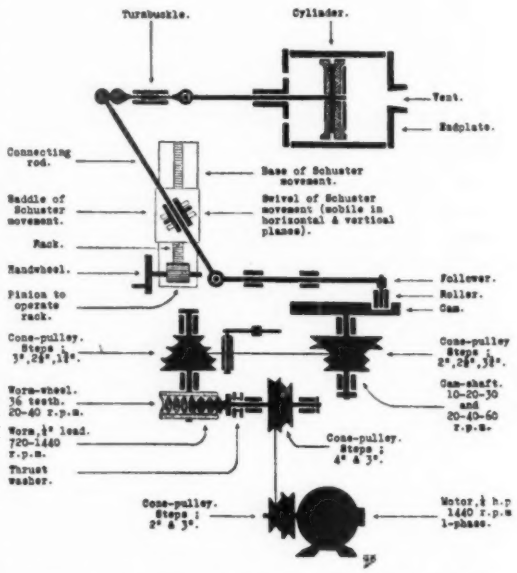

Fig. 2. Diagram to show mechanical principles of the pump.

The cam was the vital component, since it controlled movement of the piston in conformity with Fleisch's scale of time and velocity. Its design was reached in the following way. Fleisch's published tracings were measured to determine, as accurately as possible, the respiratory velocity at different times during each cycle. A time-velocity Table was thus prepared. It was converted into a time-displacement Table[5] by the method of Bevan (1945). The values so obtained were plotted with polar co-ordinates. The resulting profile, which was that of the desired cam, was then transferred to and cut out in a steel plate. The adoption of a large cam reduced the effects of minor irregularities in profile. Some difficulty was met in devising a suitable follower, free from binding couples. An internal-roller follower was found to answer best.

The follower operated upon the piston-rod through a connecting-rod which traversed an enlarged version of a Schuster movement, familiar in physiological laboratories. This movement, which is essentially a moveable fulcrum, allows the stroke of the piston-rod to be varied at will from zero to any required amount. With a cylinder of 6·000 inches bore, a stroke of 2·158 inches is required for the delivery of one litre. The maximum stroke of the prototype was 2·330 inches. A scale was inscribed upon the base of the Schuster movement to indicate settings of the fulcrum for those stroke-volumes most often required.

The cylinder was designed originally for a stroke-volume of two litres, representing the extreme of hyperpnoea in the human subject. The lightly-built prototype was, however,

[5] The assistance here of Mr. J. D. Silberberg, Dip.Mech.E., is gratefully acknowledged.

incapable of the necessary length of stroke, viz. 4·316 inches. It was therefore decided to be content with a shorter stroke and an output of one litre. The larger volume might well be adopted, however, by any future builder of the pump.

The Schuster movement is subject to severe binding stresses and must be heavily built.

A turnbuckle was interposed between the connecting-rod and the piston-rod. Its purpose is to regulate the distance between the piston and the cylinder-head at top dead-centre, producing a variable "dead-space volume" for use in future studies.

In the cylinder endplate a duct was bored to a standard taper, so that it might receive the valve or other appliance to be studied.

Oscillographic Testing of the Prototype.[6]

Introductory. The testing of the prototype set some novel problems, for which experimental methods had to be devised. Further, it was thought wise to employ methods which would apply equally to the future uses of the pump, e.g., the study of valve-design. Accordingly, the following experimental approach was envisaged.

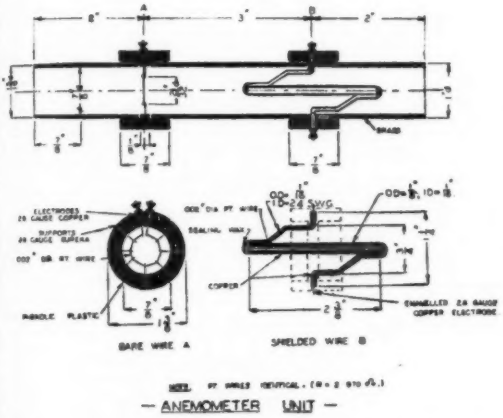

Fig. 3. Anemometer used for testing the prototype.

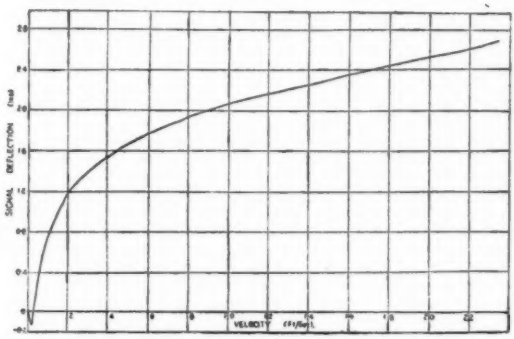

Fig. 4. Calibration-curve of the anemometer.

The flow pattern could be determined at any point of the delivery-pipe from the pump by placing there a portable recording unit. In the case of the duct, the flow recorded would represent the inflow of any valve subsequently to be inserted. After insertion of this valve, the application of the recorder to its far side would measure the outflow curve, whilst the actual movement of the valve-disk might be photographed in silhouette or otherwise. From the three instantaneous values of inflow, outflow and lifting of disk, the resistance opposed by the valve to respiration might be computed mathematically.

From this standpoint, the logical measuring device would be either: (i) a hot-wire anemometer of a special type, sensitive to the general pulse of the flow, but unaffected by local disturbance, or (ii) a piezo-electric crystal. The latter was found to be unsuitable for the very long period of pulsation, due to the phenomenon of decay of the signal: therefore, the hot-wire anemometer was selected.

[6] By B. N. Ellis.

Apparatus. As a result of experimental work carried out in the Department of Mechanical Engineering, an arrangement suitable for this kind of work has been evolved. It consists of a circle of platinum wire (Fig. 3) concentric with the pipe in which it is placed and in a plane at right angles to the flow.

The measurement of low air-speeds involves special factors with a wire of this type. If the wire is too cold, there is insufficient sensitivity over the low-speed range and, possibly, a more varied signal than with a hotter wire, because of atmospheric disturbances, such as changes in temperature. On the other hand, the phenomenon of a negative loop occurs at the low-speed end of the calibration-curve (Fig. 4) and the higher the temperature of the wire, the higher is the value of velocity at which this loop occurs. This obviously limits the temperature of the wire for use at low velocity, since, below this point, the calibration-curve is useless. A wire-temperature found experimentally to give a satisfactory result was one of approximately 70° C., and the resistance used in the bridge-circuit was calculated to give this.

The bridge-circuit used was the constant-current circuit (Ower, 1933), since this gives a direct reading on either a milliameter or a cathode-ray oscilloscope. The circuit actually used is shown in Fig. 5. A and B are identical hot wires of platinum, both exposed to flow, except that B is shielded from direct cooling-effect by means of a thin conducting sheath. The other two arms of the main bridge consist of two identical manganin resistances, the value of which equals the resistance of the hot wires when heated to the predetermined temperature of 70° C. Thus, for still air, the bridge is balanced and no current flows through the milliameter. There is, in addition, a compensating network by means of which the bridge may be balanced for varying atmospheric conditions. When this network is in use, the current in the main circuit is reduced by means of a large and normally short-circuited resistance to a value so small as to ensure that the platinum wire B is not appreciably heated.

As it was desired to obtain results in photographic form, the detector used was a cathode-ray oscilloscope of a special type.

Owing to the extremely long period of the pulsations to be studied, an especially long time-base was required. Since A.C. amplification of signals having long periods is very complicated, a D.C. amplification-unit was necessary. To facilitate visual inspection, a cathode ray tube with a long-persistence screen was provided.

Method of taking curves. Compensation for atmospheric conditions is obtained by the following adjustments to the bridge:

The main bridge is balanced by adjusting the current. This is then reduced to 1/100th of its normal value by switching-in the large resistance and the arms B and C are connected into the compensating network. B acts as a platinum resistance-thermometer and compensation is obtained by balancing with the slide-wire. This last is now offset by an amount corresponding to an excess of temperature of approximately 50 Centigrade degrees and balance is restored by adjusting the current. This heats the wire B to the required temperature above the atmospheric. The arms are now switched back into the main bridge-circuit and the large resistance is cut out. The bridge is now ready for use. The amount of offset of the slide-wire is found by trial-and-error and is that needed to give balance of the main bridge when operating at the required temperature.

For use with a cathode-ray oscilloscope, the milliameter is switched out and the Y-plates of the oscilloscope are connected between the junctions of the bridge-arms. The time-base is obtained by means of a trigger circuit actuated from the cam of the pump.

The oscilloscope is calibrated directly by fitting the anemometer unit to the outlet of an adjustable steady-flow and noting the signal-level given on the screen by various flows, as determined by a standard orifice in the steady-flow.

The anemometer-unit is then fitted to the cylinder-duct of the pump and, under the required conditions of stroke and speed, the image which appears on the screen is photographed.

A necessary precaution in this work is that calibration and measurements should follow each other immediately, owing to the need for identical atmospheric conditions and to the phenomenon of ageing in the platinum wire, which results in a change of resistance.

Correction of results. Owing to the non-linear relationship between velocity and the signal, the resulting image must be corrected. This correction involves a certain amount of skill and experience in selecting both the datum-level of the signal and the exact point at which each half-cycle concludes. The former is complicated by the formation of a negative loop, particularly at the change-over points in low-speed tests: the latter, by the presence of backlash in the mechanism of the pump. A further complication lies in the fact that the signal is always unidirectional, a reversal of velocity still giving a positive signal. Hence, at the higher speeds, the signal does not return to zero.

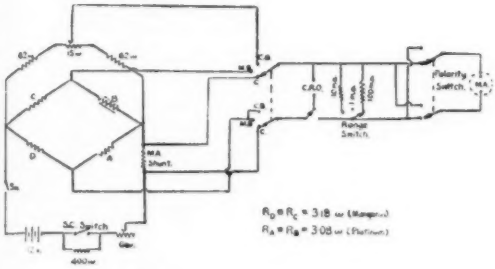

Fig. 5. Bridge-circuit for oscillography.

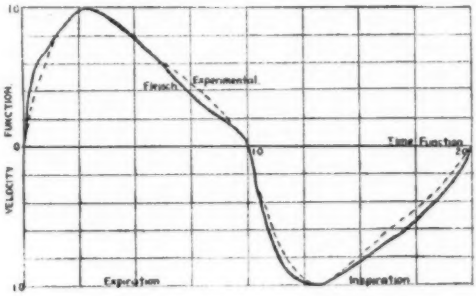

Fig. 6. Comparison of the mean inspirational time-velocity curve of the prototype with the Fleisch curve. The experimental "expiratory" phase is really an inspiratory half-cycle with its sign reversed.

A rough check upon the correctness of the selected datum-level is given, however, by comparison between the area beneath the corrected curves and the swept volume of the piston. Another check was obtained, in this particular case, by comparison of the maximal velocity with that obtained from the velocity-time relationship of the piston, as measured by piston-displacements.

Test-results. The mean experimental curve was found to follow very closely the form of the mean piston-velocity curve, as determined by measurements of piston-displacement. This showed the final result to be a true indication of the actual flow-pattern. A slight difference existed in the form of the inspirational and expirational cycles, due probably to backlash in the mechanism and to minor dissimilarity in the hand-shaped cam-profiles. In view of the latter probability, the inspirational curve (which was the less subject to backlash and which approximated the more nearly to the Fleisch curve) was chosen as the basis of comparison.

The correlation between the mean experimental curve for inspiration and the Fleisch curve (Fig. 6) is rather good. Comparison of the mean curves for each test-speed showed, however, a definite trend as the speed increased, in that the curves flattened out and the maximum occurred later in the cycle. This trend is due to the effect of the inertia of the mass of air and to the relatively greater effect of time-lag upon the signal-response as the period is decreased.

In work of this kind, however, a variation of $\pm$ 2–3 p.c. should be taken as being within the limits of error, owing to difficulty in selecting the exact centre of the trace.

The examples of worst performance (Fig. 7) were almost unique in each run, in that, for a given range of speed, usually only one stroke-volume was found to give a poor curve. All other stroke-volumes in that range usually fell within ± 1 p.c. of the mean. Even the worst curves still bore a generic resemblance to the Fleisch curve.

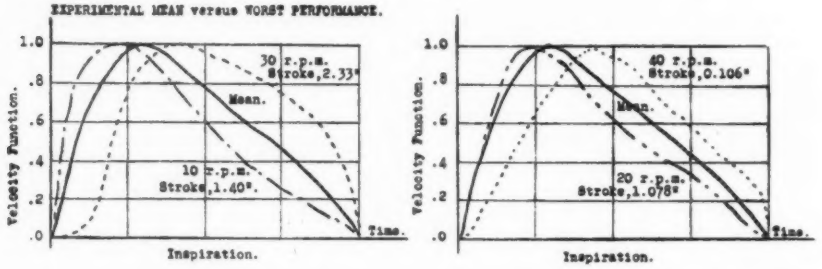

Fig. 7. Variation of the worst oscillographs from the experimental mean. The mean experimental curve (Fig. 7) is a close reproduction of the Fleisch curve. The departure from it of the worst oscillographs obtained, at various speeds and lengths of stroke, is here depicted in graphic form. A certain generic resemblance still persists.

The curves themselves were compared on the basis of position of the maximum, rather than of local variation from the mean, since in all cases the form was similar. The maximal variation, on this basis, did not exceed 10 p.c. At lower speeds, the variation was not very marked and could have been due to errors in correcting the curves. At higher speeds, however, the worst performances lay exclusively at the ends of the stroke-range, i.e., at maximal or minimal stroke. At maximal stroke, overloading of the mechanism of the pump caused the piston to stick and the movements to become irregular. This, together with the effect of increased inertia, was responsible for the variation recorded. At minimal stroke, inertia again affected the form of curve, but the problem was complicated by difficulties in interpretation.

The above effects became so great at high speeds that tests at 60 strokes per minute were quite unsatisfactory and incapable of being interpreted. Later, when the pump is redesigned to take a heavier load, it should be possible to carry out successful tests at this speed.

TENTATIVE REDESIGNING OF THE PROTOTYPE.[7]

The design which follows should eliminate various mechanical defects in the prototype, especially variation in speed at peak loads. The pump should be robust and rigid. Moving parts, whilst fitting closely, should yet work freely. Specifications for certain components, such as the cam, admit of no alternatives: others are tentative and can be varied to suit the materials and equipment at the command of the builder.

The motor (Fig. 8, A) should be of half horse-power, to give a reserve of power at full speed and stroke, whilst yet operating from a standard power-point of 10 ampères. Its speed of 1,440 revolutions per minute could be reduced in the first instance by the stepped vee-belt drive B. This, having ratios of 1 : 1 and 1 : 1·5 would reduce the speed to 1,440 and 960 revolutions

[7] By H. Adams.

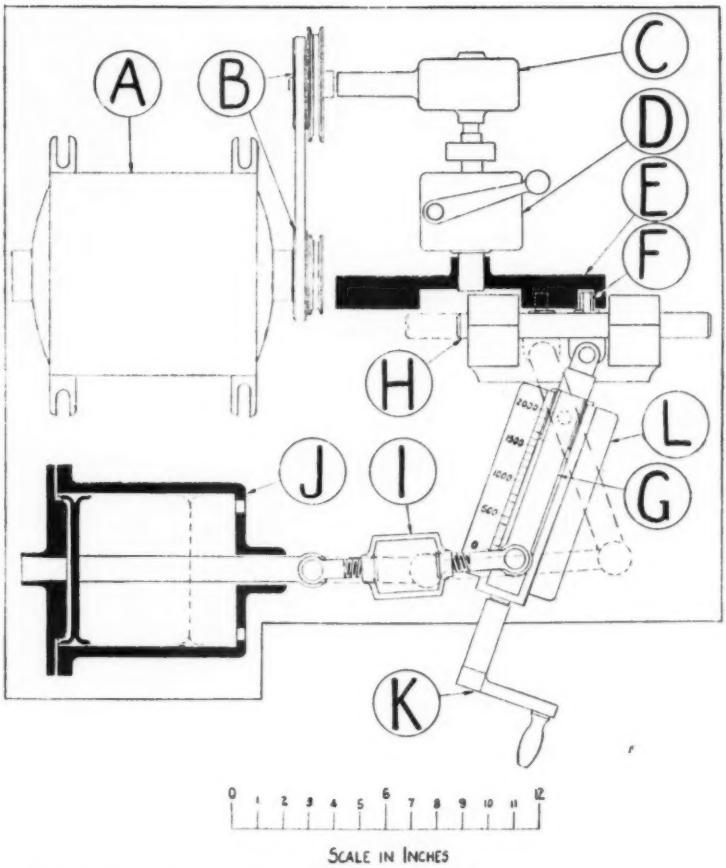

Fig. 8. Tentative re-design of the respiratory pump. This design is intended to overcome certain mechanical defects in the prototype. It uses, so far as possible, standard industrial components. The dimensions recommended may be read off from the attached scale.

per minute. Further reduction could be effected by the worm-and-wheel C, of ratio 1 : 24, thus reducing the speed to 60 and 40 revolutions per minute. The final reduction could be secured by the three-speed gearbox D. This, taken in conjunction with the speed of the belt-drive B, would give the final speeds set out in Table 1.

A light gearbox from a motor cycle or motor car could probably be adapted to the purpose, the gears being replaced should their ratios be unsuitable.

TABLE 1.

Speeds available to redesigned pump.

Gear-ratio in gearbox	Final speeds (Belt drive, 60 r.p.m.)	Final speeds (Belt drive, 40 r.p.m.)
1:1	60	40
1:2	30	20
1:4	15	10

The cam E, with its "kidney-shaped" slot, is of course the most important component of the pump. Time-displacement values for it are set out in Table 2. This Table represents intervals of one-eighth of a second over a respiratory cycle of three seconds: it is therefore plotted at polar intervals of 15 degrees over a full circle.

TABLE 2.

Time-displacement values for cam of pump.

Angle	Radius	Angle	Radius
Deg.	In.	Deg.	In.
0	2·625	195	4·313
15	2·703	210	4·169
30	2·884	225	3·959
45	3·109	240	3·743
60	3·345	255	3·530
75	3·574	270	3·327
90	3·782	285	3·157
105	3·956	300	2·994
120	4·093	315	2·860
135	4·180	330	2·746
150	4·298	345	2·667
165	4·353	360	2·625
180	4·377		

It is recommended that the cam-slot be cut on a suitable Pantosec-type profile-miller, minimizing errors by the use of the largest template within the capacity of the machine. Further accuracy could be obtained by plotting additional points. The profile obtained by the plotting of the figures in Table 2 represents that traced by the centre of the follower and due allowance must be made for the diameter of the styler wheel in preparing the template. It will be noticed that the cam is generated over a comparatively large radius in order to spread the lift and provide smooth operation.

The follower (Fig. 8, F) consists of a hardened steel roller on a spindle a quarter-inch in diameter. The spindle is attached to a cross-slide H, of section one inch square, supported by two adjustable bronze bearings. These should be set as closely together as is possible.

The connecting-rod G is hinged to the cross-slide, as shown. Its other end has a turnbuckle I, hinged between it and the piston-rod.

The fulcrum for the connecting-rod is carried on the Schuster movement. This last allows the movement of the piston to be set at will between the extremes of zero (when the fulcra of piston and connecting-rod coincide) and 4·317 inches. The last-mentioned figure represents an output of two litres from the cylinder J, which has a bore of six inches. As the throw of the cam approximates to 1·75 inches, a leverage of about 2 : 5 is needed to produce the required maximal stroke of the piston.

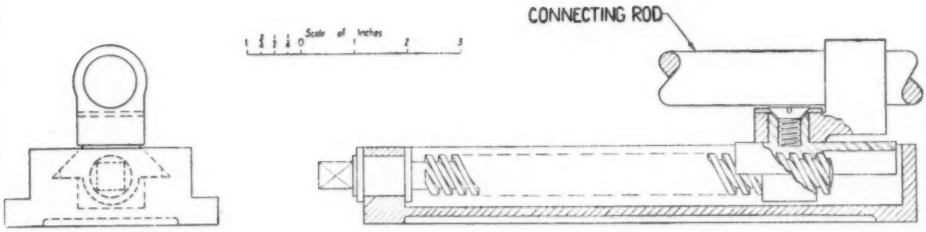

Fig. 9. Detail of Schuster movement. The dimensions recommended may be read off from the attached scale.

Details of the fulcrum of the Schuster movement are shown in Fig. 9. So that the position of top dead-centre may remain constant for the piston over its whole range of stroke, the mounting of the Schuster movement must receive careful attention. It must be set in line with the extreme inwards position of the connecting-rod, as drawn at G in Fig. 8. The extreme outwards position is shown, for comparison, in interrupted lines. The Schuster movement should be built like the top-slide of a lathe; indeed, it might well be made from such a top-slide, having a free movement of approximately six inches. The fulcrum of the connecting-rod should be mounted as low as is possible upon the Schuster movement, so as to reduce springing and consequent distortion of the Fleisch curve.

The design of piston and cylinder has been retained here as in the prototype. Other builders may find it convenient to modify the dimensions to suit whatever standard cupped washers are locally available. In this event, the stroke would need to be recalculated to ensure the desired volume of delivery. The adjustment may lie within the range of the Shuster movement: if not, the throw of the cam (but not, of course, its relative proportions) would need to be recalculated.

All components should be mounted upon a plane and rigid base-plate, otherwise binding forces may result from distortion. The base-plate might be cast from a light alloy or be fabricated by welding. In either case, it should be "normalized" before being machined.

SUMMARY.

The velocity of human respiration is not uniform, but varies throughout each cycle in the manner described by Fleisch.

The respiratory pump usual in physiological laboratories is deficient, in that it delivers at uniform velocity and not in conformity with the time-velocity curve of Fleisch.

A new cam-operated pump, designed to overcome this difficulty, is here described.

Oscillographic tests of the pump are described and show it to reproduce the Fleisch curve very closely.

Technical data are given for the construction of the new pump, mainly from standard industrial components.

Acknowledgment. Thanks are due to Professor P. L. Henderson and Acting-Professor E. J. C. Rennie, who made available the facilities of the Engineering School for this investigation.

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THE PRODUCTION OF p-AMINOBENZOIC ACID BY *STAPHYLOCOCCUS AUREUS*, MADE RESISTANT TO SULPHONAMIDES

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(Accepted for publication 6th June, 1950.)

Many workers have reported that induction of sulphonamide resistance in certain bacteria resulted in an increased production of a sulphonamide antagonist, as indicated by its appearance in the medium. The properties of this substance led to the belief that it was p-aminobenzoic acid (P.A.B.). The subject has been reviewed briefly in a recent monograph by Work and Work (1948). Lemberg *et al.* (1948), using a specific chemical method for identification of P.A.B., were unable to demonstrate increased P.A.B. production in a resistant strain of *Escherichia coli;* the excess of diazotizable amine formed under certain conditions by the resistant strain was shown to be anthranilic acid, not P.A.B. Using the same method of identification we have now shown that, under certain conditions, *Staphylococcus aureus* adapted to sulphanilamide and to sulphathiazole produces a far greater amount of P.A.B. than the non-adapted form. Continued transfer in the presence of sulphathiazole, however, produced a resistant strain which formed far less P.A.B. than the partly resistant strain, though still somewhat more than the unadapted.

Resistance to sulphonamides is apparently caused by increased P.A.B. production in some instances, but by a different mechanism in others.

EXPERIMENTAL.

Bacteriology.

The strain of organism used in experiments 1 and 2 was NRRL B 313, obtained from the School of Tropical Medicine and Hygiene, Sydney University. The characters of this strain, which are well known, were confirmed by us. Pigmentation has been retained throughout a large number of subcultures.

In experiment 3 a different strain was used. This organism, a highly pigmented strain ("N" strain), obtained from an eye infection, produced α-haemolysis on sheep blood agar, and was coagulase- and fibrinolysin-positive. Lactose and mannitol were fermented with the production of acid. The strain was pathogenic to mice. These properties, except pigment

[1] This work has been supported by grants from the National Health and Medical Research Council of Australia.

formation, were the same after induction of resistance. It was noted that after the first few subcultures in the glucose-phosphate-broth medium used in these experiments, both organisms showed a marked tendency to dissociate into non-pigmented and normally pigmented colonies.

Medium.

The composition of the culture medium was as follows:

A. KH_2PO_4, 4,500 mg.; $FeSO_4 \cdot (NH_4)_2SO_4 \cdot 6H_2O$, 50 mg.; $MgSO_4 \cdot 7H_2O$, 100 mg.; $(NH_4)_2SO_4$, 500 mg.; NaOH (Anal.), 1,120 mg.

B. Glucose, 500 mg.; nicotinamide, 0·02 mg.; pyridoxine hydrochloride, 1·25 mg.; thiamine hydrochloride, 0·0003 mg.; l-cysteine, 25 mg.

C. Veal broth (prepared by Wright's method), 10 ml. or 100 ml. (see below).

Tap water to 1 litre.

The constituents marked "A" were stored as a 40 fold concentrate and autoclaved as required. The "B" constituents were stored in the refrigerator as a 40 fold concentrate in 0·1 N hydrochloric acid, after sterilization by filtration. Fresh medium was prepared every month and stored in the refrigerator. Its pH was 7·6. This medium supported the growth of 10^9 viable units per ml. after 16 hours' incubation, from an initial concentration of 100 units per ml. given by an inoculum from a 24 hours' culture. After 16 hours a marked tendency for aggregation of the cells was observed, making viable counts at this stage somewhat unreliable.

Drug Solutions.

Sterile solutions of sodium sulphathiazole and of sulphanilamide in full medium, containing 4 mg. per ml. were prepared by adding the sulphonamide with solution A to tap water, and autoclaving one litre lots at 10 lb. pressure for ten minutes. Solutions B and C were then added. These solutions were kept in a cool place in the laboratory. 6 in. × 1 in. tubes containing 10 ml. of medium were used throughout the adaptation procedure.

Adaptation Procedure.

Experiment 1. A single colony of NRRL B 313 was selected. The medium contained 10 p.c. of broth.

TABLE 1.

Progressive diminution of growth of unadapted organism in the presence of sulphathiazole.

	Turbidity (in arbitrary units)				
	Serial transfers in 4·0 mg./ml. sulphathiazole			Drug-free control	Cells from first drug transfer in drug-free medium
Hours	First transfer	Second transfer	Third transfer		
1	0·4	0·4	no growth	0	0
2	0·6	0·6	no growth	1·4	0
3	1·4	1·0	no growth	2·0	0
4	2·0	1·4	no growth	7·0	0
5	4·4	2·0	no growth	12	0
6	6·5	2·6	no growth	17	0
7	8·7	3·0	no growth	22	0·3
8	12·6	3·0	no growth	28	2·0
9	14·5	3·0	no growth	32	3·0
10	16·8	3·0	no growth	33	5·0
24	30*	6·0	no growth	34	30

* A soluble yellow pigment appeared in the medium at this stage. Its nature was not investigated.

When the freshly isolated organism was first introduced into medium containing 4 mg. per ml. of sulphathiazole or into a similar medium containing sulphanilamide, growth was almost equal to that in the drug-free control. Growth diminished, however, in the second transfer, and in the third there was no growth. Washed cells from the first sulphathiazole culture added to the drug-free medium grew only after a lag of several hours, but attained growth equal to that of the control in twenty-four hours. This apparent increase of sensitivity to sulphathiazole is illustrated in Table 1.

In this experiment 0·1 ml. of inoculum was added to 10 ml. of medium, and transfers were made 24-hourly. Similar experiments with various drug concentrations showed that the effect was apparent with concentrations of both drugs as low as 0·1 mg. per ml. and that sulphathiazole was more inhibitory than sulphanilamide. The decrease in growth was less marked when larger inocula were used.

TABLE 2.

Growth from different inocula with various drug concentrations (44 hours).

Growth is recorded in arbitrary units of turbidity.

(a) Organism adapted to 4 mg./ml. of sulphathiazole after 16 passages.

Initial number of cells per ml.	Sulphathiazole mg./ml. 4	2	1	0·1	0
2×10^6	27	30	36	36	36
2×10^4	20	30	36	36	33
2×10^2	1	17	29	26	28
2	0	0	0	0	0

(b) Unadapted organisms cultured with sodium sulphathiazole.

Initial number of cells per ml.	Sulphathiazole mg./ml. 4	2	1	0·1	0
4×10^6	0	28	33	43	43
4×10^4	0	15	15	15	40
4×10^2	0	0	0	0	40
4	0	0	0	0	0

(c) Organism adapted to 4 mg./ml. of sulphanilamide after 16 passages.

Initial number of cells per ml.	Sulphanilamide mg./ml. 4	2	1	0·1	0
3×10^6	30	30	37	36	44
3×10^4	28	28	30	30	30
3×10^2	22	30	33	37	26
3	0	35	26	41	34

(d) Unadapted organism cultured with sulphanilamide.

Initial number of cells per ml.	Sulphanilamide mg./ml. 4	2	1	0·1	0
8×10^6	43	46	53	53	56
8×10^4	38	36	32	42	42
8×10^2	24	36	40	40	48
8	0	27	32	37	44

During this stage of our work a paper of Melchior and Klotz (1949) was published reporting a similar phenomenon with *E. coli*.

Adaptation to both sulphonamides was commenced with an inoculum of 0·1 ml. into a medium containing 0·1 mg. drug per ml. This inoculum usually contained 10^8 viable units. Three parallel series of cultures were carried out, one corresponding to each drug, and an additional one of unadapted organism as a control, which was transferred into drug-free medium at the same time as the organisms undergoing adaptation. Transfers to the succeeding series were carried out every 24 to 48 hours. For the next inoculum, we selected the culture in the highest concentration of drug, which had attained a turbidity approximately equal to that of the drug-free control.

A viable count of each inoculum was made. The remainder of the culture was centrifuged, and the cells were washed once with 10 ml. of medium on the centrifuge. After removal of the supernatant, the cells were dried over phosphorus pentoxide *in vacuo*, and later examined for production of P.A.B.

After the sixteenth transfer, the degree of adaptation was tested by examining growth with various inocula in varying drug concentrations (see Table 2).

It is seen from this Table (2a and 2b) that adaptation to 4 mg. of sulphathiazole per ml. produced an organism which grew in the presence of drug from a small initial number of 200 viable units per ml. The unadapted organism required a much larger initial number (4×10^6 per ml.) (2b).

The unadapted organism was able to grow in the presence of 2 mg. of sulphanilamide per ml. even from the smallest inoculum (2d). The adapted organism, however, was able to be transferred without diminution of growth, whereas the unadapted showed marked slowing down, from an initial number of 10^7 cells per ml. in the presence of only 0·1 mg. per ml. of sulphanilamide.

In *experiment 2* a different single colony of NRRL B 313 was adapted to sulphathiazole in medium containing 1 p.c. of broth only, with an inoculum of 0·1 ml.

In *experiment 3*, the medium again contained only 1 p.c. of broth, and 0·01 ml. of inoculum (10^5 viable units) was transferred. In this instance, the tube selected for the inoculum to the succeeding series was considerably lower in drug concentration than that showing growth equal to the control. Adaptation proceeded much more slowly than in experiments 1 and 2.

It is seen then that as a result of the adaptation process the organism had developed the ability to be subcultured repeatedly in the presence of sulphathiazole and sulphanilamide from a small inoculum.

Preparation of Large Volumes of Culture for Extraction and Estimation of P.A.B.

The cells were centrifuged off from the culture, suspended in 10 ml. of drug-free medium, centrifuged again and re-suspended in 10 ml. of fresh medium. A needle point inoculum of the suspension was then transferred to 10 ml. of fresh medium; after 16 hours' incubation this culture was used to inoculate large volumes (25 to 50 litres) in the following manner. Soluvac (1 litre) flasks containing all constituents except broth, were kept in the incubator overnight. The inoculum was then added to broth at 37° C., and a measured quantity of the inoculated broth was added to each flask; the large volume so obtained ensured more even inocula. At certain intervals flasks were removed from the incubator and small samples taken for viable counts. One fiftieth volume of conc. hydrochloric acid was then added to the bulk of the solution.

Details of the estimation of acidic ether-soluble diazotisable amine are described in a previous paper (Lemberg *et al.*, 1948). In general 20 ml. of 1 p.c. sodium nitrite were added to 1 litre of the acidified culture, followed after two minutes by 20 ml. of 5 p.c. ammonium sulphamate, and after three more minutes by 10 ml. of 10 p.c. dimethyl-α-naphthylamine in

alcohol. The naphthylamine had been previously purified by refluxing with formic acid, followed by vacuum distillation. Saturated sodium acetate solution was added until a faint cloudiness appeared. This solution was allowed to stand until next day and then completely neutralized to congo-neutrality by further addition of sodium acetate. The azo-dye was extracted with peroxide-free ether, and re-extracted from the ether by 1 p.c. sodium carbonate. The carbonate solution was neutralized with phosphoric acid and the dye again brought into ether. The dye was then extracted as the hydrochloride by 10 p.c. hydrochloric acid, and estimated spectrophotometrically in a Hilger-Nutting visual spectrophotometer, as previously described. For the calculation of the amounts of P.A.B. the extinction coefficient of the dye hydrochloride derived from 1 mg. P.A.B. in 1 ml. was assumed to be 300.

Identification of P.A.B.

A dry neutral solution of the acidic dye in ether was submitted to chromatography using B.D.H. "Alumina for adsorption analysis," previously washed with 10 p.c. hydrochloric acid and thoroughly dried at 160° C. The dye was adsorbed in a narrow band at the top of the column. On development with 1 p.c. acetic acid in ether, a sharp orange band separated and rapidly passed through the tube, leaving behind a slowly moving diffuse purple band. A second chromatograph of the dye obtained from this purple band again showed division of the dye into two fractions similar to the first chromatograph. About 70 p.c. of the original colour in the ether solution prior to chromatography was recovered from the two orange bands. The dye from the orange bands, when extracted from ether with 20 p.c. hydrochloric acid, slowly precipitated in the form of the typical crystals of the hydrochloride of the P.A.B. azo-dye. The resultant supernatant remained colourless. About 10 mg. of pure hydrochloride crystals were obtained from 25 litres of the sulphanilamide-adapted culture in experiment 1, after incubation for 100 hours. Azo-dye from pure P.A.B. showed only the orange band when chromatographed. It was noticed that the amount of dye which separated as the purple band, varied with the method of the first ether extraction of the culture. On one occasion, when the same ether was used repeatedly for extraction from a number of one litre samples, there was much less dye in the purple band fraction. The dye from the purple band produced a few typical crystals of P.A.B. azo-dye hydrochloride, in addition to amorphous material, when extracted from ether by hydrochloric acid; larger amounts of the pure crystalline dye hydrochloride were obtained when the amorphous hydrochloride was crystallized from hot alcohol by the addition of aqueous hydrochloric acid. It appears that the purple band was caused by a substance extracted simultaneously with the P.A.B. dye, which alters the adsorptive properties of the alumina for the dye, and not by the presence of another dye, derived from a different amine.

The crystals were identified as those of the azo-dye hydrochloride of P.A.B. by the following properties. They were long straight needles of blue colour with green lustre. Under the polarization microscope they showed weak pleochroism, red violet colour in a position parallel to the plane of polarization, darker and somewhat more bluish colour in a position perpendicular to it. The extinction between crossed nicols was straight. The neutral azo-dye prepared from the hydrochloride crystallized from light petroleum in the typical orange leaflets of the P.A.B. dye, and its melting point (220–221° C. on the copper block) showed no depression with that of the P.A.B.-dye.

The larger part of the acidic ether-soluble diazotizable amine is thus P.A.B.

Effect of Adaptation to Sulphonamides on the Production of P.A.B.

Table 3 (experiment 1) shows that the cultures of the organisms adapted to 4 mg. per ml. of sulphathiazole or to the same concentration of sulphanilamide in 16 transfers form an appreciable amount of ether-soluble acidic diazotizable amine during the phase of active growth

(8–14 hours) in sulphonamide-free medium, while the unadapted organism forms only traces. Up to 33 hours the amount in the adapted cultures does not rise further, while that in the unadapted culture reaches about the same level. Finally, after 100 hours, very large amounts of the amino compound are found in the adapted cultures, but very little in the unadapted culture.

TABLE 3.

Production of P.A.B. by Staph. aureus *NRRL B 313 (Experiment 1) (10 p.c. broth).*

			Adapted to 4 mg./ml. drug in 16 transfers			
	Unadapted		Sulphathiazole		Sulphanilamide	
Hours of incubation	Viable count × 10^6	P.A.B. μg. per litre	Viable count × 10^6	P.A.B. μg per litre	Viable count × 10^6	P.A.B. μg. per litre
0	0·002	0	0·0025	0	0·0025	0
4	—	0	0·06	0	0·062	0
6	—	0	1·0	0	12·5	trace
8	80	0	30	0	370	13
10	—	trace	400	trace	640	21
12	—	trace	1,200	20	900	20
14	1,300	trace	1,200	29	1,250	25
26	500	15	500	29	100	25
33	—	26	—	27	—	27
47	—	—	—	52	—	124
100	—	10	—	400	—	123

TABLE 4.

Production of P.A.B. by Staph. aureus *NRRL B 313 (Experiment 2) (1 p.c. broth).*

	After 13 transfers				After 26 transfers			
	Unadapted		Adapted to 0·4 mg./ml. sulphathiazole		Unadapted		Adapted to 0·2 mg./ml. sulphathiazole	
Hours of incubation	Viable units × 10^6	P.A.B. μg. per l.	Viable units × 10^6	P.A.B. μg. per l.	Viable units × 10^6	P.A.B. μg. per l.	Viable units × 10^6	P.A.B. μg. per l.
0	10	0	17·5	6	0·16	0	2	0
14	—	—	—	—	38	7	9	10
16	—	—	—	—	41	7	30	14
18	—	—	—	—	62	6	—	—
20	150	0	100	500	53	6	—	—
24	145	23	116	505	—	—	65	20
29	—	—	—	—	—	—	—	12
36	—	—	—	—	—	—	300	63
40	—	—	—	—	400	10	—	—
44	370	20	365	850	—	—	—	—
48	—	—	—	—	230	11	—	—
52	—	—	—	—	—	—	215	27
68	363	17	325	1,210	—	—	—	11
86	—	—	—	—	—	11	—	—
110	—	—	—	—	—	10	—	21

In experiment 2 the same organism has been used as in experiment 1, but adaptation to sulphathiazole has been carried out more slowly and to a smaller degree. Growth is slower, since the medium contains only 1 p.c., not 10 p.c. of broth, and in spite of a far larger inoculum, the viable count at the end of the growth period is smaller than in experiment 1. Table 4 shows that under these conditions the organism adapted to 0·4 mg. of sulphathiazole per ml. produces very large amounts of P.A.B., and that the rise can be noticed earlier than in experiment 1. After 13 further transfers, adapting the organism to 2·0 mg. of sulphathiazole per ml., the production of P.A.B. has become far smaller, though still larger than that by the unadapted.

Table 5 (experiment 3) shows that the "N" strain made resistant to 2·0 mg. of sulphathiazole per ml. after 50 transfers produces a very large amount of P.A.B., far more than the unadapted organism. The unadapted organism of this strain produces somewhat more P.A.B. than the unadapted organism of the other strain used in experiments 1 and 2. Slow adaptation to sulphathiazole has not caused the decrease of P.A.B. production found on prolonged adaptation of the other strain.

TABLE 5.

Production of P.A.B. by the "N" strain after 50 transfers in sulphathiazole, final resistance to 2 mg./ml. of drug (Experiment 3).

Hours of incubation	Unadapted P.A.B. μg. per l.	Adapted P.A.B. μg. per l.
0	0	0
68	55	1,600

TABLE 6.

Production of P.A.B. at 100 hours at various stages of adaptation.

No. of transfers	1	7	8	9	10	11	12	16
Sulphathiazole mg./ml.	0	0·1	0·1	0·1	0·5	0·5	1·0	4·0
P.A.B. μg. per l.	0	13	91	118	153	260	299	400

TABLE 7.

The effect of Reductone on the Diazotization of P.A.B.*

P.A.B. mg./l.	Reductone mg./l.	P.A.B. mg./l. extracted as azo dye	p.c. P.A.B. diazotizable
1	0	0·80	100
0·1	0	0·08	100
1	1	0·55	70
0·1	0·1	0·83	100
1	10·0	0·14	17
0·1	1	0·082	99

* Reductone was prepared by the method of v. Euler and Martius (1933). Reaction mixtures were allowed to stand overnight before diazotization.

For the experiment tabulated in Table 6, dried culture of the NRRL B 313 strain, mentioned previously, have been suspended in medium, and a small inoculum has then been transferred with a loop into 250 ml. lots of medium with 10 p.c. broth. These are incubated for 100 hours, and P.A.B. is then determined as before. Table 6 shows that there is a continuous rise in production of P.A.B. related to the stage of adaptation of the organism. In this series there is no indication of a decrease of production of P.A.B. on prolonged adaptation. Only the late production of P.A.B. has been measured in this instance and adaptation has been rapid, with only 16 transfers.

DISCUSSION.

It has been shown above that adaptation of *Staphylococcus aureus* to sulphathiazole or to sulphanilamide, leading to increased resistance, is associated with a high yield of ether-soluble acidic diazotizable amine in the medium. This amino compound has been identified with p-aminobenzoic acid by specific chemical methods.

Considerable variations in the appearance of P.A.B. in the medium have been found, depending on the strain, the composition of the medium, and on the adaptation procedure.

Under the conditions of Table 4 (slow growth in medium containing 1 p.c. broth) partly adapted NRRL B 313 strain produced far more P.A.B. than more fully adapted strain. This indicates that *Staphylococcus* becomes first resistant to sulphonamides by an increase of P.A.B. production, but later by a different mechanism which shortcircuits the reaction inhibited by sulphonamides. In *E. coli* the second way of developing resistance appears to develop immediately.

The development of the shunt mechanism does not seem, however, to be a general phenomenon with *Staphylococcus*. No decrease of P.A.B. production has been found with prolonged adaptation of the "N" strain, or in 16 transfers of the NRRL B 313 strain, making it highly resistant to sulphathiazole or sulphanilamide, when the organism grew rapidly in medium containing 10 p.c. broth (Table 6).

In the last instance the rate of appearance of P.A.B. in the medium (see Table 3) is quite different from that observed under the conditions of slow growth summarized in Table 4. The appearance of P.A.B. during the phase of active growth (8–14 hours) is little marked, but increased synthesis of P.A.B. by the organism is apparent from its late appearance in the medium. Since this occurs many hours after the viable count has begun to decrease, the phenomenon can hardly be explained by P.A.B. accumulating in the organism when the rate of P.A.B. utilization and conversion into non-diazotizable products has decreased, while the rate of P.A.B. synthesis is maintained. It appears more likely that non-diazotizable conversion products, e.g. folic acid or its precursors undergo a breakdown during the lysis of the organisms. We have been unable to detect P.A.B. in the cells of adapted or unadapted organisms removed during

the growth phase, even after hot alkaline (pH 9) or acid (pH 2) hydrolysis. Apart from pteroylglutamic acid, pteroic acid or a condensation product of P.A.B. with glucoreductone are non-diazotizable. Koft and Sevag (1949) have observed partial breakdown of pteroylglutamic acid to P.A.B. on prolonged incubation in sterile medium, but the yield of P.A.B. was only of the order of 1 p.c. Glucoreductone is assumed to be formed in the growth phase (O'Meara, McNally and Nelson, 1947; Forrest and Walker, 1948; Bell, Cocker and O'Meara, 1949). We have, therefore, studied the reaction between P.A.B. and reductone produced according to the method of v. Euler and Martius (1933). Our results (Table 7) show that the diazotizability of P.A.B. is diminished by reductone to a considerable degree only if P.A.B. and reductone are both present at relatively high concentrations. Since various reductones exist, and since their concentration as well as of P.A.B. in the growing bacterial cell are unknown, the possibility cannot be excluded that during the phase of active growth practically all free P.A.B. disappears in this condensation. We have, however, been unable to discover glucoreductone in rapidly growing cells by the colour reaction with o-dinitrophenol.

SUMMARY.

Two strains of *Staphylococcus aureus*, NRRL B 313 and a strain obtained from an eye infection, have been adapted to sulphathiazole and to sulphanilamide, and the appearance of p-aminobenzoic acid in the medium has been studied.

An apparent increase of sensitivity of the unadapted organisms during the early transfers is described.

The drug-adapted organisms produced a larger amount of ether-soluble acidic diazotizable amine than the unadapted organisms. This was identified with p-aminobenzoic acid by a specific chemical method.

The rate of appearance of P.A.B. in the medium depends on the strain, the adaptation procedure, and the composition of the medium. Under certain conditions a fully adapted organism produced less P.A.B. than a partly adapted one. This may be explained by the development of a shunt mechanism. Under conditions of rapid growth P.A.B. may not appear in large amounts during the phase of active growth, but it does so many hours after the viable count has begun to decrease. This is probably due to a formation of P.A.B. from non-diazotizable compounds, such as pteroylglutamic acid, pteroic acid, or the condensation product of P.A.B. with reductone, during the lysis of the organisms.

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MELIOIDOSIS IN SHEEP IN QUEENSLAND

A DESCRIPTION OF THE CAUSAL ORGANISM

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(Accepted for publication 23rd May, 1950.)

Mallcomyces pseudomallei has been isolated from fatal cases of a disease in sheep in Queensland. There is no record of the previous isolation of this organism in Australia, and this is believed to be the first report of its occurrence in sheep.

In this paper the characteristics of the organism are described. An account of the outbreak will be published elsewhere.

Malleomyces pseudomallei was first isolated by Whitmore and Krishnaswamy in 1912 from the bodies of vagrants brought to the mortuary at Rangoon. Whitmore (1913) described the organism under the name *Bacillus pseudomallei*. It was renamed *Bacillus whitmori* by Stanton and Fletcher (1921). Topley and Wilson (1929) placed the organism tentatively in the genus Pfeifferella as *Pf. whitmori* along with the closely related glanders bacillus, *Pf. mallei*. Bergey *et al.* (1939) classify it as *Mallcomyces pseudomallei*. The disease produced in man and animals by *M. pseudomallei* is called "melioidosis" (Stanton and Fletcher, 1921).

In man infection is usually fatal, 2 only of 83 cases recorded up to 1932 having survived (Stanton and Fletcher, 1932). The symptoms are varied and simulate those of typhoid, cholera, plague, lobar pneumonia, miliary tuberculosis, pleurisy, malaria, enteric fever and infective endocarditis (Stanton and Fletcher, 1932; Strong, 1945).

Autopsy reveals the presence of abscesses, these being most common in lungs, liver and spleen though they may occur in any organ except the brain, in which they have not been seen.

Very few chronic cases of the disease have been reported. Most human infections run an acute septicaemic course with death occurring from 3 days to 4 weeks (Couture, 1935).

Melioidosis in either man or animals or both has been reported from Burma, Federated Malay States, Indo-China, Ceylon, Netherlands East Indies, Celebes, Guam, Siam, Madagascar.

In the human case reported in England by Grant and Barwell (1943), the infection was evidently contracted in the Far East.

Bacteriological Findings.

Isolation.

On the 9th May, 1949, a sample of pus from an abscess in the lung of a sheep was forwarded to this laboratory. A pure culture of *M. pseudomallei* was obtained on blood agar streaked with the pus.

On 7th June, 1949, a sample of pus taken from another sheep in the same flock was received. This also yielded a pure culture of *M. pseudomallei.*

Morphology and Staining.

The organisms occurred as short rods with straight axes, parallel sides and rounded ends.

When freshly isolated the organisms appeared singly, in pairs end to end, and in small groups. They stained Gram negative with marked bipolarity. After several subcultures the bacilli were mostly grouped in bundles, and the bipolarity was less pronounced.

The organisms were actively motile in broth cultures incubated at 22° C. or 37° C.

No spores or capsules (Muir's Stain) were demonstrated in pathological exudate or in culture. The organism was not acid fast (decolourized by 25 p.c. HCl). A single tuft of flagella at one pole was shown by Kirkpatrick's stain.

Cultural Characteristics.

Growth was abundant on all ordinary media, although 48 hours at 37° C. was required on solid media to produce a characteristic colony shape.

Nutrient agar plate. At 37° C. after 24 hours' incubation, colonies were up to 1 mm. in diameter. After 48 hours' incubation, there was an abundant growth of smooth, cream coloured, umbonate colonies 1–2 mm. in diameter with an entire edge. On plates left at room temperature after 48 hours' preliminary incubation, colonies attained a maximum size of 1 cm. in diameter about a week later. When plated on nutrient agar after several subcultures in nutrient broth, the smooth colonies were translucent greyish-white, and by transmitted light were iridescent. In addition some rough crinkled colonies were present.

Gelatin stab. Stratiform liquefaction occurred after 6 days at room temperature. There was a thick layer of growth between the liquefied and unliquefied portions and no surface pellicle was formed. Complete liquefaction required 2-3 months at room temperature.

Nutrient broth. After 24 hours' incubation at 37° C. a dense turbidity developed and a thin surface pellicle was formed. This thickened on further incubation and after several days a slimy deposit gathered, leaving the supernatant moderately clear. The pellicle was still present.

Glycerol agar. When freshly isolated on 1 p.c. glycerol agar the organism appeared after 48 hours at 37° C. as smooth umbonate cream coloured colonies, which did not alter in form with further incubation.

When subcultured on 5 p.c. glycerol agar, and incubated for 48 hours at 37° C., the colonies were round, cream coloured, with a slightly wrinkled centre (Fig. 4). If the plates were then left at room temperature for 2 to 3 days the growth became heaped up and very wrinkled, ridges of growth extending radially to the periphery from a high central peak (Figs. 2, 3).

10 p.c. sheep's blood agar. After 24 hours at 37° C. there were smooth, translucent, greyish-white colonies 1 mm. in diameter, with a round regular edge.

After 48 hours at 37° C. the colonies were a dirty white, 2–4 mm. in diameter, with a raised centre, flat periphery and regular edges (Fig. 1). Some colonies had concentric raised rings of growth.

Haemolysis was present around the primary inoculum. After three days at 37° C. the medium had turned brown.

Fig. 1. *M. pseudomallei* on chocolate agar after 3 days at 37° C.

Fig. 2. *M. pseudomallei* on 5 p.c. glycerol agar after 2 days at 37° C. and 7 days at room temperature.

Fig. 3. *M. pseudomallei* on 5 p.c. glycerol agar after 3 days at 37° C. and 6 days at room temperature.

Fig. 4. *M. pseudomallei* on 5 p.c. glycerol agar after 2 days at 37° C.

Loeffler's serum slope. Abundant growth occurred and after three days at 37° C. liquefaction commenced. After 15 days digestion was almost completed.

Potato. After 48 hours at 37° C. growth was thick and cream coloured. After 3 weeks at 37° C. the colour was similar to that of milk coffee.

MacConkey's agar (difco). Growth was evident after 24 hours and after 48 hours the colonies were red, opaque, raised, dome shaped, about 2–3 mm. in diameter.

No growth was obtained on Difco S.S. agar.

Cultures on all media had an earthy aromatic odour.

Colonial Forms.

The colonial form of the organism differs on different media.

On 10 p.c. sheep's blood agar the organism regularly appears in the form of the original isolate, namely off-white colonies, with a round regular edge, flat periphery and raised centre (Fig. 1). This form has been designated type A.

Our type A colony appears to be similar to one described by Nicholls (1930) as an "intermediate" form. However, two isolations, namely from the testis and spleen respectively of two experimental guinea-pigs, gave rise to yellow, convex, moist round colonies with a regular edge. This form has been reproduced with successive platings on blood agar.

On 5 p.c. glycerol agar the usual type of growth is the very wrinkled, sometimes heaped up growth similar to that described by Stanton and Fletcher (1932) (Figs. 2, 3). Some variation in the degree of wrinkling occurs as can be seen from Figs. 2 and 3. On occasions no wrinkling has occurred.

Nutrient agar plates have not given rise to wrinkled colonies, the growth usually being similar to that of type A on blood agar, but degrees of variation have occurred from this to a round regular, convex type of colony.

Biochemical Reactions.

The freshly isolated strain fermented the following carbohydrate media without the formation of gas, after incubation for the following periods at 37° C.: glucose, 24 hours; maltose, lactose and mannitol, 48 hours; laevulose, galactose and trehalose, 9 days; arabinose, mannose, glycerol and dulcitol, slightly in 24 days.

Xylose, rhamnose, sucrose, raffinose, adonitol, sorbitol were not fermented.

After six successive subcultures in nutrient broth glucose only was fermented.

Eight months after isolation the above strain fermented only glucose, fructose, galactose on the seventh day and trehalose after 14 days.

A decline of fermentative ability was found also in cultures recoved from experimentally infected animals inoculated with the original culture. These cultures failed to ferment many of the carbohydrates fermented by the original isolate.

Nitrates were reduced to nitrites, indole was not produced. Methyl red, Vosges-Proskauer and urease tests were negative. Litmus milk was clotted in 5 days and digestion of the clot followed.

Catalase and ammonia were positive, H_2S production negative. Methylene blue was reduced after 18 hours.

The organism was killed by heating to 56° C. for 10 minutes. Anaerobically very slight growth was present after 10 days.

Pathogenicity.

Guinea-pigs.

A wide range of doses of broth cultures of *M. pseudomallei* (from 1 ml. of 10^7 dilution to 1 ml. undiluted) has been inoculated into guinea-pigs. Both intramuscular and intraperitoneal routes have been used with similar results. All cases have been fatal, death occurring from 18 hours to seven days, most often within 48 hours, after inoculation.

The animals became weak and reluctant to move. In those which survived more than two or three days there was a milky ocular and nasal discharge.

The post-mortem findings have been fairly constant. The spleen has been the most noticeably affected organ, being greatly enlarged, and studded with numerous small abscesses. The kidneys usually appeared normal. The liver and pancreas almost always were studded throughout with small abscesses. The lungs and the omentum often contained abscesses. The testes, although not enlarged, were often adherent to the scrotal sac, with abscesses present on the tunica vaginalis and surrounding tissue. Large abscesses formed at the site of inoculation. Enlarged lymph nodes, especially those nearest to site of inoculation, have been found.

Mice.

Intraperitoneal inoculation of 0·1 ml. of broth culture of the organism proved fatal within 48 hours. Autopsy disclosed abscesses in the liver, lungs and spleen, the latter being greatly enlarged.

Sheep.

An adult Merino ram (M1) was inoculated intramuscularly in the right thigh with 4·0 ml. of a 24 hour broth culture on 27th May, 1949. It died 12 days later.

The temperature was raised 24 hours after inoculation and fluctuated between 104·7° F. and 107·1° F. until death. Complete suppression of appetite was followed by marked loss of weight. The conjunctival and scleral blood vessels became engorged on the fourth day and an ocular discharge appeared on the eighth day. Later this discharge became thick, yellow and tenacious. The site of inoculation became swollen and lameness appeared first in the inoculated limb and later in both hind limbs. A thick, yellowish, tenacious nasal discharge was present about the seventh day and gradually increased in volume. On the eleventh day the animal had difficulty in breathing, apparently due to partial nasal obstruction, and he was weak and reluctant to move. On the twelfth day he was prostrate. An autopsy was done half an hour later after death.

Autopsy. At the site of inoculation there was a lesion about 12 × 8 cm. in the base of the thigh muscles with central necrosis and surrounding suppuration. Oedema and fibrosis involving muscle and connective tissue had extended down the muscle to the tendon and upwards slightly.

The superficial inguinal and internal iliac lymph nodes on the inoculated side were enlarged and contained firm light-orange inspissated pus replacing almost all of each node.

The spleen contained several nodules up to 1 cm. in diameter raised above the capsule.

The liver was swollen and congested but no focal lesions were present.

The heart and kidneys were normal.

The lungs were studded throughout with small abscesses up to 0·5 cm. in diameter. The bronchial and mediastinal lymph nodes were normal as were the mouth, tongue and larynx.

The nasal mucosa on the septum and turbinate bones was swollen and dark red and was studded with numerous white abscesses some of which had brown necrotic centres and had ulcerated. Most of these were about 3 mm. across. A purulent discharge was present.

The organism was cultivated from the lesion at the site of inoculation, the turbinate bone, adrenal gland and the spleen but not from the lungs, kidneys, liver and testes.

Subsequently three more sheep were inoculated with *M. pseudomallei.*

A Merino wether about 1 year old (No. 574) was inoculated intravenously with 1 ml. of overnight broth culture. It was killed *in extremis* 14 days after inoculation.

There was an acute polyarthritis but no lesions in the viscera. In the nasal cavity lesions similar to those in the previous sheep, M1, were present. The organism was recovered from kidney, urine, nasal lesions and affected joints.

A Merino wether about 1 year old (No. 728) was inoculated supraconjunctivally with a few drops of broth culture. The sheep was killed a month later after having shown symptoms of encephalitis for a few days. No macroscopic lesions were found and the organism was not recovered.

Another Merino wether about 1 year old (No. 569) received 1 ml. of broth culture by mouth. No symptoms were seen and the animal was sacrificed two months after inoculation. The only lesions found were erosions on the articular surfaces of the limb joints. The organism was not recovered from any organs or joints.

DISCUSSION.

There is close agreement between the characteristics of the organism described and those of *M. pseudomallei* found in the literature.

Readiness of staining with ordinary dyes and the pronounced bipolarity exhibited by the organism are features mentioned in all descriptions of morphology seen. Stanton and Fletcher (1932) remarked that the bipolar forms looked like closed safety pins, though the large elements 15–20μ in length mentioned by them have not been observed by us.

The marked rough, wrinkled growth on 5 p.c. glycerol agar, one of the cultural characteristics described by other workers and regarded as characteristic of the organism, was noted in our cultures and this feature is shown in the monograph by Stanton and Fletcher (1932).

An earthy aromatic odour was present in all our cultures and was quite characteristic. Mention of a distinctive odour in cultures has been made by Pons (1927) and Stanton and Fletcher (1932).

The fermentation reactions are not of great value in identification owing to the decline in fermentative power upon subculture and ageing.

Other biochemical findings such as liquefaction of gelatin, clotted milk, coagulated serum, reduction of nitrate, negative indole test, negative methyl red, Vosges-Proskauer tests, positive urease, catalase, ammonia production, reduction of methylene blue are in agreement with those of Pons (1927), Stanton and Fletcher (1932), Topley and Wilson (1929).

Production of H_2S was negative. Topley and Wilson (1929) list this as $\pm$ in their description.

The oxidase test described for gonococci yielded positive results in the hands of Miller *et al.* (1948), but our strain was negative using dimethyl phenylene diamine hydrochloride.

The pathogenicity for guinea-pigs and the lesions produced conform closely to the descriptions of Stanton and Fletcher (1932).

Abscesses in the pancreas following intraperitoneal injection is a feature that has not been mentioned by other workers, but has been a constant finding with our strain.

The appearance of a Strauss reaction in guinea-pigs following intraperitoneal inoculation has been a regular finding of other workers with this organism. In our experience the testicles of guinea-pigs inoculated intraperitoneally

with our strain of the organism have not become swollen. However, another feature of the Strauss reaction, namely abscesses on the tunica vaginalis, has occurred.

The only previous mention of *M. pseudomallei* in connection with sheep is that of an experimental infection set up by Stanton and Fletcher (1932). The sheep died in 6 days with miliary nodules in the lungs. No other particulars were given.

SUMMARY.

Malleomyces pseudomallei has been isolated from fatal infections in sheep in Queensland.

The morphological, cultural, biochemical characters and pathogenicity are recorded.

This is believed to be the first record of a natural occurrence of melioidosis in sheep, and is the first known isolation of the organism in Australia.

Acknowledgments. I am grateful to Dr. S. T. Cowan, Curator of the National Collection of Type Cultures, who confirmed the identification of the organism.

I should like to thank also, Dr. J. Legg, Mr. A. K. Sutherland and Mr. G. C. Simmons, for their helpful advice in the preparation of this paper, and Mr. W. J. Sanderson to whom I am indebted for the illustrations.

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AN INVESTIGATION OF THE CAPSULO-RENAL REFLEX

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(Accepted for publication 27th May, 1950.)

The nerve supply of the capsule of the kidney has been described as a reticular network of nerve fibres which constitute a specialized type of end organ in the capsule, these end organs being sensitive to the physiological stimulus of changes in capsule tension. This network has been described by several writers as distinct from the vasomotor supply to the vessels of the capsule (Smirnow, 1901; Kümmel, 1925; Stöhr, 1924; Lehmann, 1926).

Decapsulation of the kidney to relieve anuria in cases of severe nephritis was suggested by Kümmel on the basis that it removed these nerves, thus destroying the capsulo-renal reflex. Fischer (1926, 1930) made extensive researches into the capsular innervation and the specific physiological function of these specialized nerves which he called the "nervii proprii of the kidney capsule". His theories as to their importance in decapsulation of the kidney were supported by physiological as well as histological research.

An increase in kidney volume by raising the tension of the capsule activates the capsular end organs which send afferents to the cord synapsing with the renal vasomotor efferents from T9-L1 (Eppinger, 1921; Kümmel, Fischer). The vasomotor reflex decreases the kidney volume when the capsule tension is increased, by constriction of the renal arterioles. The converse also appears to be true with a decrease in capsular tension. This theory forms, at present, the main basis for decapsulation in severe cases of nephritis with anuria or oliguria. Decapsulation, by relieving markedly increased capsular tension resulting from the congested nephritic kidney and also by destroying the afferent part of the capsulo-renal reflex which was carrying a "morbid" number of stimuli, relieves the renal arteriolar spasm reflexly induced. Hence the oliguria is relieved and the ischaemia of the kidney is reduced, removing the risk of permanent kidney damage.

Szabo (1948) has described a renal vasomotor reflex similar to that described by Fischer. Fischer's work has not received a great deal of attention and it was thought desirable to repeat his work in attempting to demonstrate a capsulo-renal reflex. His experiments, which he performed on the dog, have been repeated on the rabbit (with the exception of the crossed and reno-renal reflex).

Method.

The rabbit was the experimental animal used and oral paraldehyde (1·7 ml./kg. body weight) the basal narcotic, administered one hour previous to operating. With this dosage very little, if any, ether was needed during the operation. We endeavoured to work in as light a state of surgical anaesthesia as possible since too deep a state could interfere with the proper functioning of the reflex.

The changes in kidney volume were recorded by paper kymograph, and the oncometer used for registering these changes was always a closed unit. Mechanical and faradic stimulation were applied to the capsule by two fine, rigid wires passing through two holes in the oncometer which were afterwards sealed with vaseline (Fig. 1). The left kidney was used for the experiments since it was easier to approach than the right and consequently surgical interference was small.

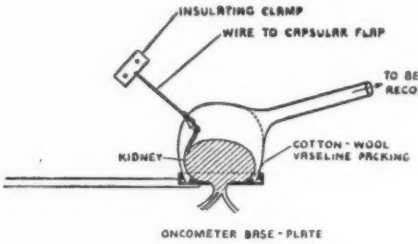

Fig. 1. Cross section.

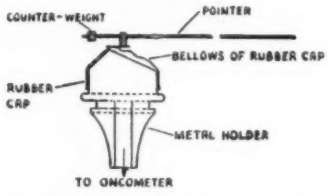

Fig. 2. Cross section of bellows recorder.

The kidney was approached through an antero-lateral abdominal incision continued by a muscle split into the peritoneal cavity. The gut was then packed off and the kidney carefully separated from the loose perirenal fat and fascia, leaving it enclosed in its true capsule. The base plate of the oncometer was moved into position, the renal vessels passing through a slit in it. The thin renal vessels were protected from the metallic edges of the base plate by cotton wool soaked in vaseline. A flap of capsule approximately one centimetre by one and a half centimetres was dissected from the broad surface of the kidney, the flap being attached near the hilum of the kidney. This flap corresponded closely to the flap used by Fischer in his work. The two fine wires were attached by hooked ends to the free border of the capsule flap, and their free ends passed through the holes in the glass top of the oncometer which was then fixed to the base plate. The free ends of the wire were held separate from one another by a clamp of insulating material. By this means mechanical or electrical stimulation could be applied to the capsule with the kidney inside a closed oncometer and as the oncometer was made of glass it was possible to watch the kidney during the experiment.

The recording of changes was carried out by means of a bellows-type recorder. The bellows recorder consists of a rubber cap on a metal holder; one side of the cap is fixed to the edge of the holder to form a hinge for a piece of celluloid stuck to the free part of the cap: movements of the hinged celluloid due to changes in kidney volume are magnified by a long pointer (Fig. 2). We were able to obtain sufficient sensitivity with this to record a clear trace of fluctuations of kidney volume caused by heart beat and respiration. One tenth of a millilitre change in kidney volume produced a deflection of 1·8 cm. of the pointer on the drum.

A cannula was inserted into the left carotid artery and a simultaneous recording of the arterial pulse was taken on the drum.

It is necessary to mention several precautions which we had to take when carrying out the experiment. The base plate of the oncometer was carefully positioned to avoid interference with the renal vessels, particularly the veins. There was no interference with the gut or any part of the body whilst the experiment was in progress. Care was needed in dissecting up the flap of capsule to avoid interference with its nerve supply.

Over a dozen rabbits were operated upon before any reliability could be placed upon the results obtained.

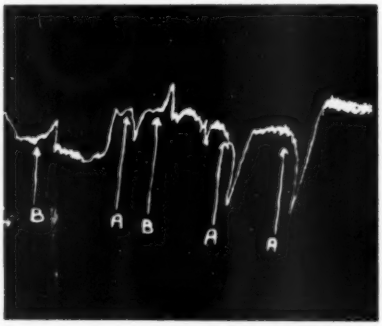

Fig. 3. Changes in kidney oncometric trace due to tension stimulus to capsule type (a).
A = increase in capsule tension.
B = relaxation of capsule tension.

RESULTS.

The type of stimulus and the results obtained from each type will be discussed separately for each type of stimulus.

Mechanical Stimulus.

(a) The first method of imparting a physiological type of tension stimulus to the capsule was to exert a gentle traction in the direction of the wires. The results obtained were uniform. There was an immediate fall in volume of about 0·1 ml. on all occasions when the capsule tension was increased, and an increase in kidney volume of the same magnitude when the tension was decreased. The immediate nature of the response was suspicious and the stimulus was repeated on the capsules after the rabbits had been killed, giving identical results. The conclusion was that these changes were caused by mechanical alterations in the shape of the kidney which occurred independently from the capsulo-renal reflex. There was no alteration in the blood pressure of the rabbit, nor was there any delayed significant change in kidney volume (Fig. 3). This experiment was carried out on seven rabbits.

(b) The second method of applying mechanical stimulus to the capsule was to approximate the ends of the wires distal to the flap; this caused the ends attached to the capsule to diverge, thus stretching it. By watching through the oncometer an appropriate degree of tension could be applied (Fig. 4). Although this type of stimulus was carried out on eight rabbits there was never any significant alteration in kidney volume (Fig. 5).

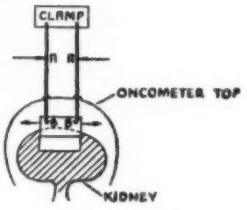

Fig. 4. By pushing the wires together at A–A the hooked ends B–B attached to the capsular flap are caused to diverge (the holes in the oncometer acting as fulcra) and the capsule flap is thus stretched by a sideways stimulus.

Faradic Stimulus.

Two main types of electrical stimulation were used. It was not possible to determine from Fischer's paper the exact frequency or strength of stimulus used so a wide range was covered.

(a) The Harvard Inductorium was run from a 2-volt battery and attached to the wires. This coil gives impulses at an approximate frequency of 50 per second at a peak voltage of 5–500 volts depending on the position of the coil. The peak voltage used in these experiments was from 5–50. In a series of eight rabbits in which this stimulus was used there was never

any significant alteration in kidney volume or blood pressure. On several occasions with the stronger stimulus there was a small diminution in volume probably from leakage of the impulses to the vessels at the hilum.

(b) There was a possibility that these impulses were not of the correct strength or frequency to stimulate these capsular end organs so an apparatus was used in which the frequency could be varied from 1/sec. to over 40/sec. This was first tested on a frog's leg preparation and the strengths necessary to produce a slight muscle twitch and to produce tetany of the muscle was determined. Varying strengths and frequencies between these limits were used. Fig. 6 shows a series of such stimuli. No appreciable change in volume was observed in any of four rabbits despite the fact that the stimuli were repeated many times. Great care was taken to ensure that a sufficient flap of capsule was always raised and the hooked ends of the wires which acted as point electrodes were watched through the oncometer to ensure that they were attached to the capsule.

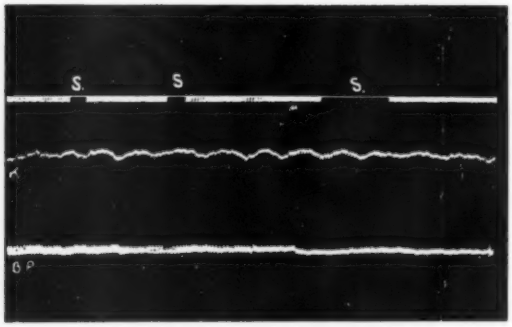

Fig. 5. Kidney oncometric (K) and arterial (B.P.) recordings with tension stimulus type (b) to capsule—denoted by S.

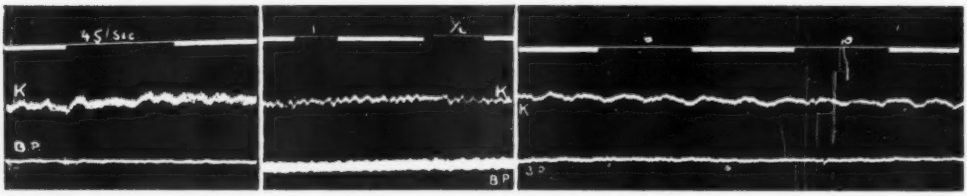

Fig. 6. Kidney oncometric (K) and B.P. recordings with faradic stimulus type (b). The figures denote the impulse frequency/second.

DISCUSSION.

No changes in kidney volume were obtained which were of any significance with either mechanical or faradic stimulus to the capsule. That the apparatus was sensitive enough can be seen from the magnitude of the kidney traces shown. Any change in volume would cause a marked alteration to the trace.

Thus it can be said that in a total of twenty-two rabbits in which these experiments were performed successfully, we were unable to demonstrate the presence of a capsulo-renal reflex in any of them. These rabbits were of varying weight, age, and sex and under varying depths of surgical anaesthesia, generally being kept as light as was possible.

The significance of these results may be discussed as follows:

It is well known that species' differences exist amongst the mammalia for various physiological processes; this difference exists even between such species as the Carnivora and the Rodentia. It would appear that the discrepancy that exists between our results and Fischer's can be explained only by a capsulo-renal reflex existing in dogs but which is not present in rabbits. (It is interesting to note that Fischer did attempt to experiment with rabbits but abandoned them because he considered their capsules too delicate to use, so there is no information on this subject in his paper.) If the possibility of a species' difference be conceded, the existence of a capsulo-renal reflex in man cannot be assumed, as it is at present.

Abehouse (1945) quotes the theories of Kümmel and Fischer as those most likely to explain the benefits obtained by decapsulation, this benefit being obtained by destruction of the reflex and consequent removal of the arteriolar spasm produced by its overaction. It can be argued that the benefits obtained by decapsulation are from the removal of a non-expanding and tightly constricting capsule which is mechanically interfering with the blood supply to the kidney. This view was held by some writers (Harrison, 1896; Lloyd, 1917).

It is important to state that the existence of the "nervii proprii" in man is not precluded by these results, but only the existence of a capsulo-renal reflex. Decapsulation, by removal of these sensory end organs can still give relief in cases of nephralgia, as has been claimed by Fischer.

Similarly, when considering the benefits obtained from local infiltration of the sympathetic chain in cases of anuria it can be argued that the relief is due to removal of spastic tone of the renal arterioles by paralysis of the sympathetic efferents, this spastic tone being first produced by a local substance liberated by a toxic or nephritic kidney.

SUMMARY.

The capsulo-renal reflex described by Fischer as it exists in dogs has been investigated. This reflex could not be demonstrated in rabbits. The importance of these findings as indicating a species' difference in the occurrence of the reflex has been discussed. A capsulo-renal reflex is not considered necessary in explaining the beneficial results obtained from decapsulation, sympathectomy or procaine block of the sympathetics to the renal vessels.

Acknowledgments. I wish to thank Professor Sir Stanton Hicks for his valuable advice, and Mr. Mark Shaw for his technical assistance during the experiments.

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End